SPECIES DELINEATION OF TWO IMPERILED WILD GINGERS (*ASARUM CONTRACTA* AND *A. RHOMBIFORMIS*) USING ECOLOGICAL, MORPHOLOGICAL, AND MOLECULAR TECHNIQUES

A Thesis

by

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ABSTRACT

SPECIES DELINEATION OF TWO IMPERILED WILD GINGERS (*ASARUM CONTRACTA* AND *A. RHOMBIFORMIS*) USING ECOLOGICAL, MORPHOLOGICAL, AND MOLECULAR TECHNIQUES (May 2008)

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Asarum contracta and *A. rhombiformis* are closely related species and both are in need of protection. Floral differences between *A. contracta* and *A. rhombiformis* have been inconclusive in separating them as distinct taxa and there is no published ecological or molecular analyses of *A. contracta* and *A. rhombiformis*. For conservation purposes, more data about species boundaries are needed.

Two questions are asked in this study. First, does the morphology, ecology and molecular data support separation of *A. contracta* and *A. rhombiformis* as distinct species? Second, does the LEAFY gene have phylogenetic utility in elucidating relationships in the complex, or more broadly in the genus *Asarum*?

Morphological differences between *A. contracta* and *A. rhombiformis* were determined using external and internal floral characteristics. The analysis of floral differences between *A. contracta* and *A. rhombiformis* using t-tests and Wilcoxon tests resulted in nine floral characteristics that differed significantly.

Ecological differences were examined first by determining the locations of *A. contracta* and *A. rhombiformis* in relation to each other and in relation to river drainages. Elevation data and co-occurring plant species were obtained. The location and elevation data did not consistently separate *A. contracta* and *A. rhombiformis.* The co-occurring plant species did not result in a significant difference in community structure, according to the Sorenson's Index of Community Similarity.

DNA extracted from the leaves of *A. contracta* and *A. rhombiformis* and one individual from each species within the "*Hexastylis* clade" were used in analysis of the second intron of the LEAFY gene to examine the genetic differences between *A. contracta,* and *A. rhombiformis* and their relationship to other members of the "*Hexastylis* clade". The LEAFY gene exhibited a 31bp indel shared between the "*Hexastylis* clade" and "*Heterotropa* clade" within *Asarum,* but did not resolve relationships within the "*Hexastylis* clade" or between *A. contracta* and *A. rhombiformis*.

Based on the significant morphological differences, the continued recognition of *A. contracta* and *A. rhombiformis* as distinct taxa is recommended to the conservation community. The results of this study imply that ecological parameters did not determine these species boundaries, and the LEAFY gene did not capture the genetic differences between *A. contracta* and *A. rhombiformis*. Therefore, additional ecological factors and other genes should be explored to concretely support or negate the recognition of *A. contracta* and *A. rhombiformis* as distinct taxa.

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INTRODUCTION

Taxonomy of *Asarum*

Taxonomic relationships in the genus *Asarum* L. (*Aristolochiaceae* Jussieu), a group of approximately 90 species, have been debated for two centuries. Rafinesque recognized *Hexastylis* as a separate genus from *Asarum* in 1825 based on a number of species in the southeastern United States that had glabrous, persistent leaves and flowers that were glabrous on the outer surface. Blomquist (1957) reaffirmed *Hexastylis* as a distinct genus but concluded that further study, including more Asian species, would clarify the generic circumscription. Apparently unknown to Blomquist, Araki's (1953) systematic analysis of the genus *Asarum*, based on North American, European and Asian species, embedded *Hexastylis* within his concept of *Asarum*. Soltis (1984), using karyotype analysis, found that species within both *Asarum* and *Hexastylis* did not differ, but that *Asarum* differed from *Hexastylis*. Kelly (1997) utilized morphological characters in conjunction with nuclear DNA sequences to examine phylogenetic relationships within the genus *Asarum* sensu lato. Morphological data were used to clarify lineages within *Asarum* (Kelly 1998) and molecular information was useful in clarifying relationships between closely related species (Kelly 2001). The combined molecular and morphological data collected by Kelly supported Araki's depiction of a single genus *Asarum* that contains two clades; *Asarum* + *Geotaenium* and *Asiasarum* + *Hexastylis* + *Heterotropa* (Figure 1). The previously recognized genus *Hexastylis* is therefore included here as a clade in the genus *Asarum* (Araki 1953, Whittemore et al. 1993, Kelly 1998, Kelly 2001, Neinhuis et al. 2005, Wanke et al. 2007).

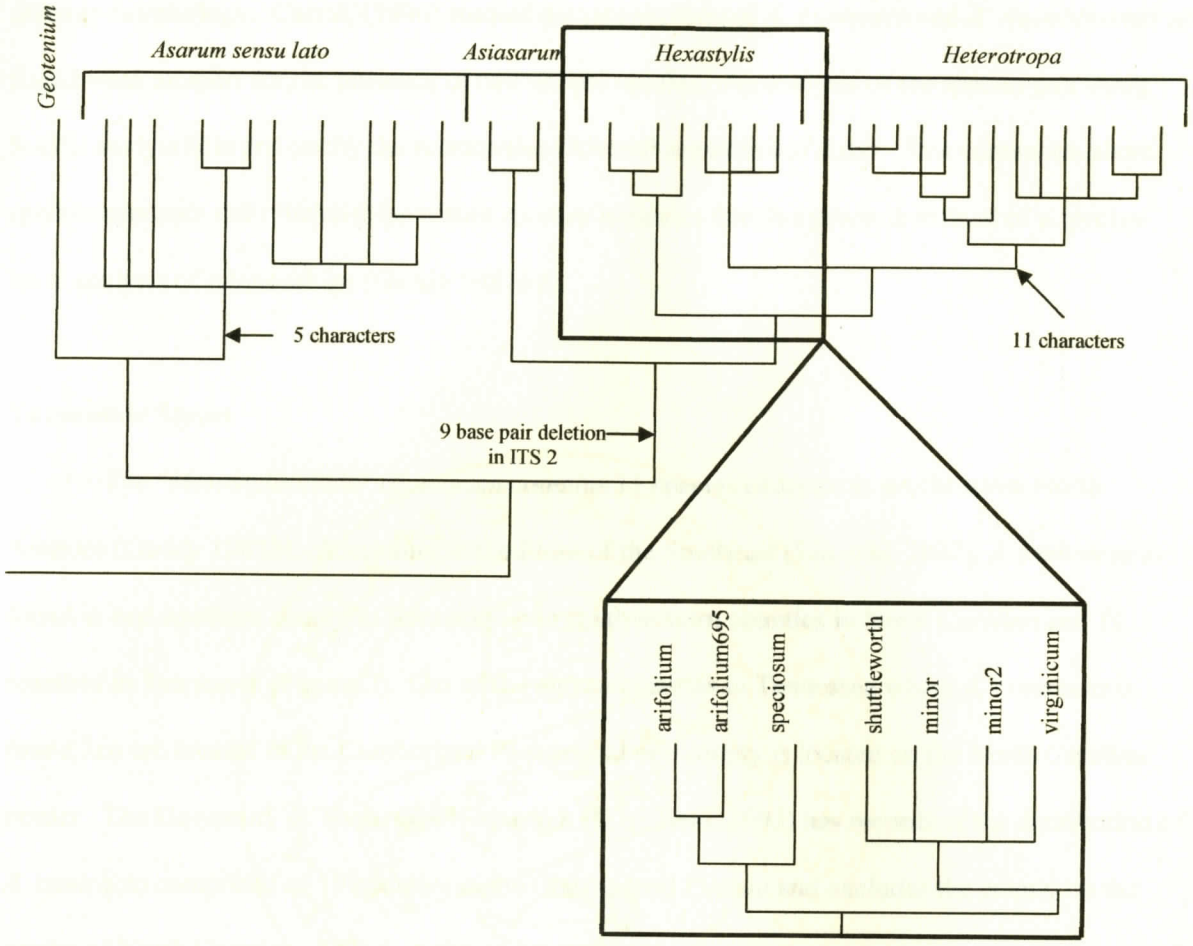

Figure 1: Representation of Kelly's (1998) phylogeny based on molecular (ITS) and morphological data.

Within *Asarum* there are questions surrounding the taxonomic relationships of many species. For instance, *Asarum shuttleworthii* var. *harperi* Gaddy has a growth form that was recognized as distinguishing it as a unique species, *A. harperi* Gaddy, but it has been more recently recognized as a variety of *A. shuttleworthii* (Britten and Baker) Small (Gaddy 1987*a*). In contrast, *A. marmoratum* Piper, a California endemic that previously had been considered a variety of *A. hartwegii* S. Wats., has been recently determined to be a unique species (Mesler and Lu 1990). Gaddy (1986) recognized *Asarum rhombiformis* Gaddy as distinct from *A. contracta* Blomquist based on floral morphology.

However, Barringer (1993) concluded that *A. rhombiformis* was a variety of *A. contractum* based on all over morphology. Carroll (1996) studied the morphology of *A. contracta* and *A. rhombiformis* and found weak support for the presence of two unique species. An analysis of the species pair using RAPD analysis did not clarify the relationship (Murrell *unpublished data*). This contention about species concepts and relationships within *Asarum* indicates that this group is in need of a species-level analysis of relationships (Gaddy 1987*b*).

Taxonomic Status

The "*Hexastylis* clade" of *Asarum* contains 11 species endemic to southeastern North America (Gaddy 1987*b*). According to the Flora of the Southeast (Liu et al. 2007), *A. contracta* is found in one southern county in Kentucky, two southwestern counties in North Carolina and 11 counties in Tennessee (Figure 2). Out of the eleven counties in Tennessee where *A. contracta* is found, ten are located in the Cumberland Plateau and one county is located on the North Carolina border. The University of Tennessee Herbarium (U of Tenn. 2007) has records of the distribution of *A. contracta* comprised of 10 counties in the Cumberland Plateau and excludes the county on the border of North Carolina. This disparity suggests that the *A. contracta* population near the border of North Carolina was mis-identified, though the specimens were not examined to clarify this disparity. The geographical range of *A. rhombiformis* is limited to five counties in North Carolina and one county in South Carolina (Figure 3) (Liu et al. 2007). The range of *A. contracta* and *A. rhombiformis* overlap in Buncombe and Henderson Counties in North Carolina. The counties where *A. rhombiformis* is documented and *A. contracta* is not known to occur include Transylvania and Burke Counties in North Carolina and Greenville County in South Carolina.

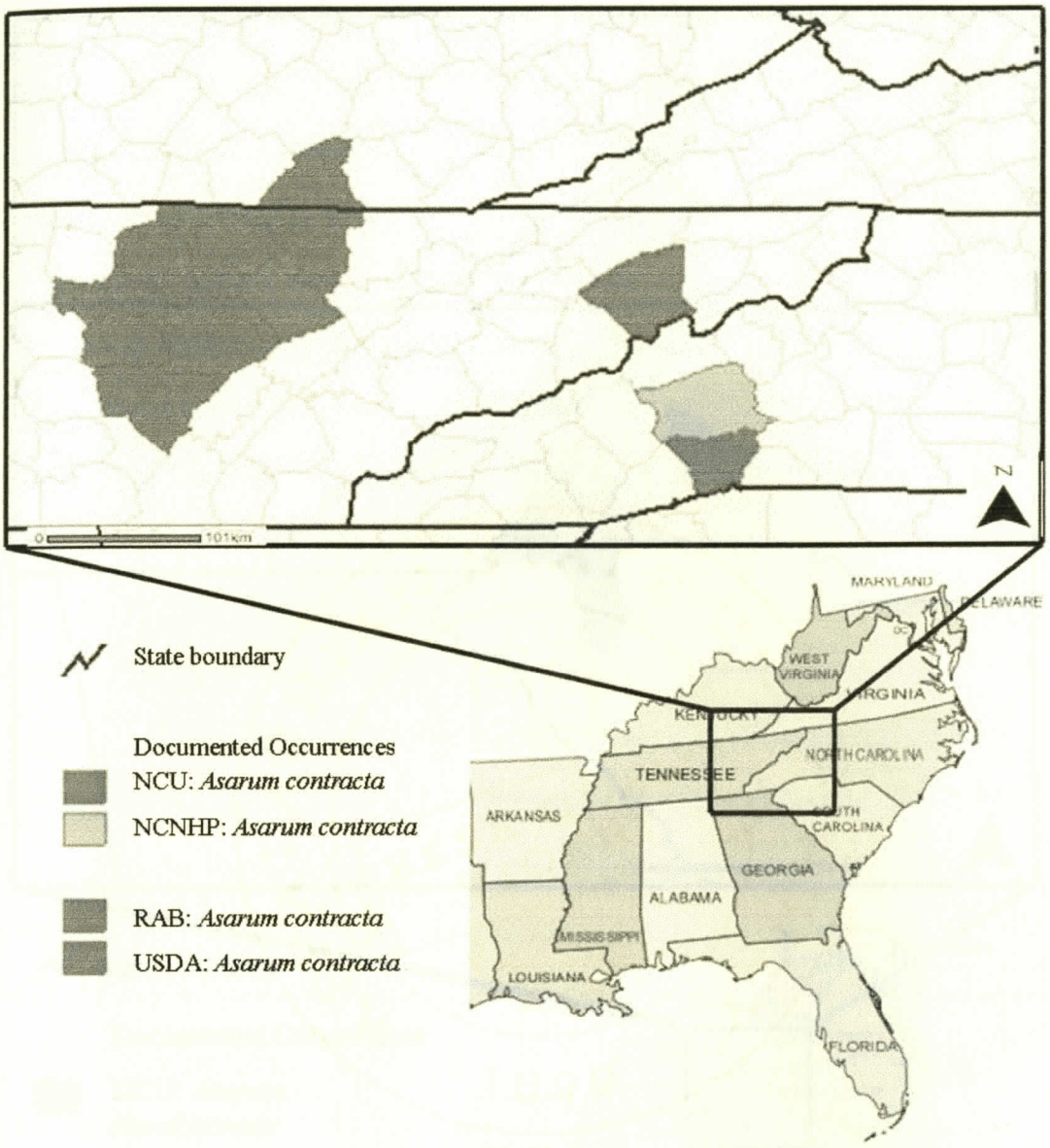

Figure 2: The known range of *Asarum contracta*. Modified from the Flora of the Southeast (Liu et al. 2007).

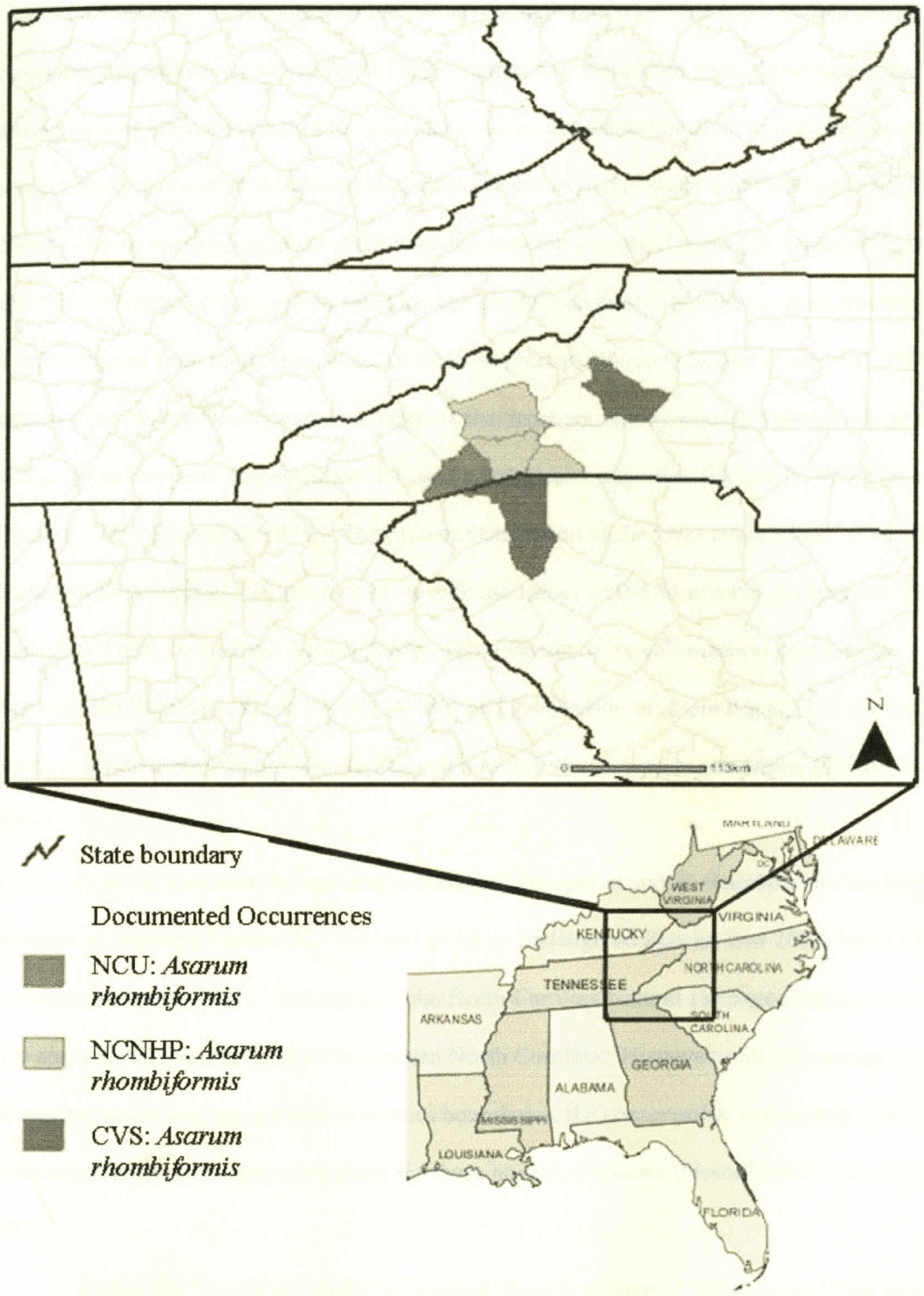

Figure 3: The known range of *Asarum rhombiformis.* Modified from the Flora of the Southeast (Liu et al. 2007).

Advancements in molecular techniques have provided another way of extrapolating past community distribution. Molecular findings suggest that there were multiple refugia in the southeast during the last ice age instead of the previously suspected exclusion of all vegetation except for the refuges south and east of the glacial maximum (Watts 1970, Gonzales and Hamrick 2005, Soltis et al. 2006). Due to the small amount of fossil pollen data for woodland herbs (Delcourt 1979, Delcourt and Delcourt 1994) the migration since the last glacial maximum for herbs such as *Asarum* often has to be estimated from the pollen record of trees that occupy the same habitat (Cain et al. 1998). It is known from pollen cores and genetic markers that trees commonly sharing habitat with *Asarum* found refuge in the Southern Appalachians during the last glacial migration (Delcourt 1979, Delcourt and Delcourt 1994, Cain et al. 1998). The present distribution of the "*Hexastylis* clade" (Otte 1977, Gaddy 1987b) suggests that it may also have found refuge in the Southern Appalachians (Davis 1983, Cain et al. 1998, Griffin and Barrett 2004). The difficulty of re-colonization posed by the Appalachian Mountains could explain the disjunct distribution of *A. contracta*, if *A. contracta* had a western and an eastern range prior to glaciation as did *Maianthemum canadense* (Griffin and Barrett 2004).

In North Carolina *A. contracta* is listed as endangered and *A. rhombiformis* has been recently elevated to threatened status (US Fish and Wildlife Endangered Species List 2002; Laura Gadd, *personal communication*). According to the North Carolina Natural Heritage Program records, these two species share similar habitats in western North Carolina. However, without appropriate evidence to support taxonomic recognition of species boundaries, the conservation community lacks a firm foundation to create conservation plans for these imperiled species (Nesom 2005, Whittall et al. 2006).

Within the "*Hexastylis* clade" of *Asarum*, there is a range of variation in flower and leaf morphology. According to Whittemore et al. (1993) *A. contracta* and *A. rhombiformis* have rhombic-ovoid calyxes that are conspicuously tapered above the middle, puberulent inside with lobes ranging from 3-8 mm wide and leaves crowded at the rhizome apex. *Asarum rhombiformis* is distinguished

from *A. contracta* by having well-developed reticulations inside the calyx that range from 1.5-2 mm in height, while *A. contracta* may either lack internal reticulations of the calyx tube or they may range from 0-1 mm in height. *Asarum rhombiformis* also has a superior ovary while *A. contracta* has a 1/3 inferior ovary. Therefore, one of the questions this study seeks to address is whether the differences found between *A. contracta* and *A. rhombiformis* merit recognition as two different species.

Carroll (1996) performed a study on *A. arifolia*, *A. contracta* and *A. rhombiformis*. The goals of his study were to locate new populations of *A. contracta*, to determine the species boundaries of putative hybrids found to be intermediates between *A. arifolia* and *A. contracta* and to examine the relationships between *A. contracta* and *A. rhombiformis*. Some segregation of *A. contracta* from *A. rhombiformis* was found in two of the principle components analysis (PCA) based on the floral morphology (Carroll 1996). However, the separation of *A. contracta* and *A. rhombiformis* was not complete and further research was needed.

With the disjunct distribution of *A. contracta* and the possibility of hybridization between *A. contracta* and *A. rhombiformis*, it is important to determine the environmental variables that could serve as barriers to reproduction. Geographical features can split a species' range and act as a key factor in speciation (Barraclough and Vogler 2000). Ecological niche partitioning has been shown to occur with species sharing habitats with close proximity to each other (Gram and Sork 2001, Fuller et al. 2007, Queenborough et al. 2007), thus there may be ecological factors affecting gene flow (Rundle and Nosil 2005, Remington and Robichaux 2007) between *A. contracta* and *A. rhombiformis*. As ecology may be a cause of adaptive radiation (Schluter 2000) or be a barrier to reproduction between *A. contracta* and *A. rhombiformis* (Langerhans et al. 2007), ecological analyses were included in this study to determine if significant ecological differences could be found.

Species Debate

Scientists have debated what defines a species (Mesler and Lu 1990, Avise and Wollenberg 1997, Sites and Crandall 1997, Baum 1998, Hey 2001) and thus how to separate taxa. The value of

the taxonomic rank of species is a highly debated with many different species concepts defining species in different ways (Mayr 1991, Nixon and Wheeler 1990, Wiens and Servedio 2000, Hendrixson and Bond 2005). Some view a species definition as a hypothesis (Sites and Crandall 1997, Baum 1998) that only differs in the way that the specific hypothesis is worded (Hey 2001) but "share the general ontological view that species are lineages" (Hendrixson and Bond 2005). A confounding issue for defining species is the occurrence of hybridization and lineage sorting (Hewitt 2001). A species that is defined by the cohesion species concept is "the most inclusive population of individuals having the potential for phenotypic cohesion through intrinsic cohesion mechanisms" including genetic exchangeability and demographic exchangeability (Templeton 1989). Due to the seemingly inclusive nature of the debate and operational utility of the cohesion species concept (CSC) I align my philosophy most closely with Templeton's (Templeton 1989, 2001) species concept.

My study considers a species to have reproductive barriers that keep it from breeding with other species, which eventually creates a distinct lineage separate from other lineages by containing characters that are passed on to offspring and not shared with other species. Ecological, morphological and genetic factors may contribute to, or create the reproductive barriers that separate the lineages. Until now, morphological and ecological studies have been unable to differentiate *A. contracta* and *A. rhombiformis*. This study applies molecular techniques in addition to morphological and ecological characteristics to a taxonomic problem in an attempt to determine if there are additional characters that support the separation of these two enigmatic species.

Phylogenetic Use of Genes

Nuclear genes have a higher rate of evolution than genes found in the chloroplast and mitochondria (Hewitt 2001, Small et al. 2004), while chloroplast genes have been found to be five times less likely to have substitutions than nuclear genes (Wolfe et al. 1987, Sang 2002). The higher rates of substitution found in nuclear genes make them more useful at low taxonomic levels (Sang 2002, Small et al. 2004, Whittall et al. 2006). Nuclear genes also offer phylogenetic information from

both maternal and paternal parents (Small et al. 2004). Therefore, a nuclear gene was chosen to explore the species level relationships between *A. contracta* and *A. rhombiformis*. One individual from each species within the "*Hexastylis* clade" was also included in the analysis to enable the exploration of potential hybrid parentage and to explore the phylogenetic utility of the gene.

The LEAFY (*lfy*) gene is a low-copy nuclear gene comprised of two introns and three exons (Frohlich and Meyerowitz 1997, Frohlich and Parker 2000, Aagaard et al. 2005, Dong et al. 2005, Dornelas and Rodriguez 2006). The second intron of *lfy* represents a non-coding area that is variable, flanked by two highly conserved exons (Frohlich and Meyerowitz 1997, Shu et al. 2000). The conserved nature of the exons facilitated the development of degenerate primers (Frohlich and Meyerowitz 1997) that have been used across several families to explore evolutionary relationships. The size of the *lfy* second intron varies widely in taxa with a length of 83 bp found in *Peperomia* Agardh to a length over 4600 bp in *Platanus* Jussieu (Frohlich and Meyerowitz 1997). Within Rosaceae Jussieu the second intron varied from 0.3 kb to 1.4 kb (Oh and Potter 2003). This variation in length demonstrates the variability of the intron. In addition to length variation, the sequence of the second intron of *lfy* also varies widely enough to produce many characters useful in phylogeny reconstruction at low taxonomic levels (Frohlich and Meyerowitz 1997, Howarth and Baum 2005). The second intron of *lfy* has been found to be more variable than *waxy*, ITS or cpDNA in several studies (Oh and Potter 2003, Smith and Baum 2006).

Initial studies found only one copy of *lfy* in angiosperms, but recent studies have found *lfy* to have multiple copies (Shu et al. 2000, Archambault and Bruneau 2004, Aagaard et al. 2005, Howarth and Baum 2005). Other nuclear genes such as GBSSI or SPB2 were originally thought to be represented only once in angiosperm genomes and have since been found to have multiple copies (Archambault and Bruneau 2004). Functioning duplicate copies of *lfy* were found in seven families within Lamiales using different sets of primers (Aagaard et al. 2005), while only one copy was found in *Cedrela fissilis* Jussieu and *Lotus japonicus* L. using the Southern blot technique (Dong et al. 2005, Dornelas and Rodriguez 2006). The multiple copies of *lfy* found in subfamily Caesalpinioideae

(Fabaceae Lindley) varied greatly in length as well as in sequence (Archambault and Bruneau 2004). The variation within *lfy* alleles in three of 49 Iochrominae (Solanaceae Jussieu) species was interpreted to be caused by those individuals having a hybrid origin (Smith and Baum 2006). The *lfy* gene has not been examined in *Asarum* and, due to its taxa dependent variability, this study will serve as an exploration of the phylogenetic utility of the this gene for *Asarum*, while enabling a species level exploration of taxonomic relationships.

Purpose of This Study

The primary aim of this study was to test the null hypopthesis that *A. contracta* and *A. rhombiformis* are distinct taxa and the operational hypothesis that *A. contracta* and *A. rhombiformis* differ significantly enough to be considered distinct taxa. To determine if ecological requirements represent a barrier to the reproduction of *A. contracta* and *A. rhombiformis* the ecology of the species was examined. Based on fresh material Gaddy (1986) concluded that the shape and details of the perianth differ between *A. contracta* and *A. rhombiformis*. Other studies have not been able to show that floral differences between *A. contracta* and *A. rhombiformis* differ significantly (Barringer 1993, Carroll 1996). Therefore, a more robust morphological analysis was performed. The *lfy* gene was used to evaluate the relationship between *A. contracta* and *A. rhombiformis*. The secondary aim of this study was to determine the utility of the *lfy* gene for the resolution of species relationships within the "*Hexastylis* clade".

MATERIALS AND METHODS

Ecological Methods

Eight populations of *A. rhombiformis* and three populations of *A. contracta* in North Carolina were sampled (Appendix A). The populations of *A. contracta* that were used in this study were located near or within populations that had been previously recorded by the North Carolina Natural Heritage Program (1996) as, HC 001, HC 003, HC 004, and HC 006. Therefore, these served as the labels used for the different populations. *Asarum rhombiformis* sites have also been recorded by the North Carolina Natural Heritage Program and the *A. rhombiformis* populations used in this study are HR 012, HR 010, HR 008, HR 007, HR 006, HR 004, and HR 001. While attempting to locate HR 002, a population of *A. rhombiformis* undocumented by the Natural Heritage Program (1996) was found and is represented by HR New.

Location, elevation and vegetation were classified within each population to test if ecological processes support the distinctness of *A. rhombiformis* and *A. contracta*. Within each population, one 20 m x 50 m plot was constructed and the co-occurring vegetation was sampled using the protocols and techniques established by the Carolina Vegetation Survey (Figure 4) (Peet et al. 1998). Location and elevation data were obtained using a handheld Garmin GPS unit.

The mean number of co-occurring plant species found in all of the *A. rhombiformis* populations were compared with the mean number of plant species found in *A. contracta* populations. The mean number of co-occurring plant species for *A. contracta* and *A. rhombiformis* were tested for significant differences using a t-test. A t-test was also performed using the elevation data. Vegetation was identified using Newcomb's Wildflower Guide (1977) and then checked using The Flora of the Carolinas, Virginia, Georgia, and Surrounding Areas (Weakley 2007). Vegetation

differences were inferred using a Sorenson's Index of Community Similarity. The equation for Sorenson's Index was C = 2j/(a+b) where C was the coefficient of community, j = number of co-occurring species common to both *Asarum* species, a = number of species found in *A. rhombiformis* populations and b = number of species found in *A. contracta* populations.

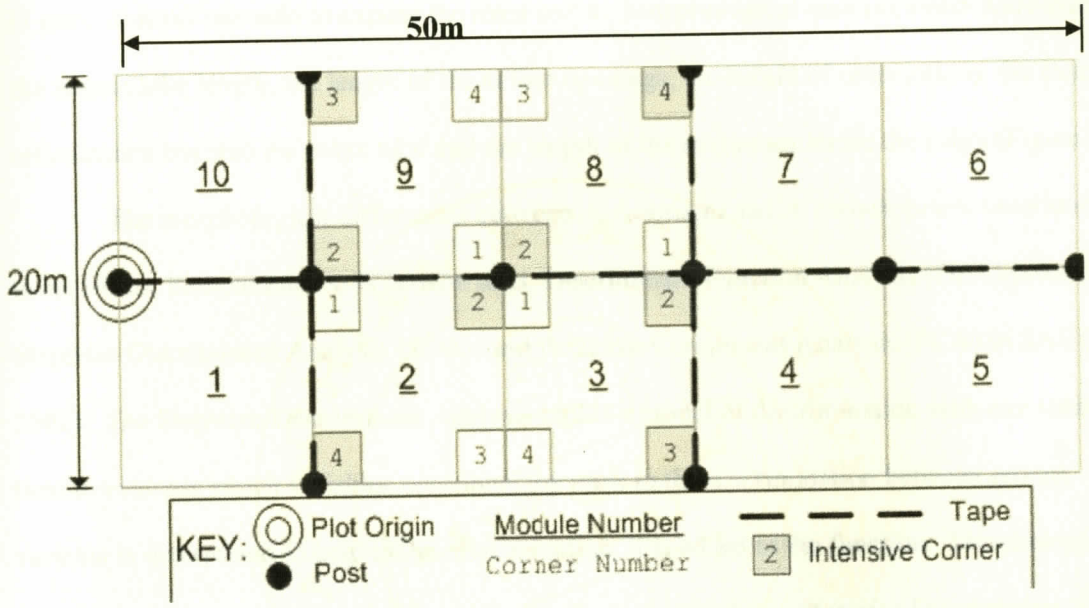

Figure 4: Plot design used for vegetation sampling recreated from the Carolina Vegetation Survey (http://cvs.bio.unc.edu/protocol/cvs-eep-manual-v4_lev3-5.pdf) 2006.

Morphological Methods

Within eight populations of *A. rhombiformis* and four populations of *A. contracta* in North Carolina, three flowers from three different plants were removed for morphological analysis and species verification. The flowers of *A. rhombiformis* and *A. contracta* lack petals but possess a calyx. Length and width measurements were taken using the protocol for measuring morphological characteristics of *A. rhombiformis, A. contracta*, and *A. arifolia* established by Carroll (1996). Length measurements were taken (Figure 5) of the total length of the calyx (FF), the length from the "neck" to the base of the flower (FE), and from the widest point of the flower to the base of the flower (FD).

Width measurements were taken (Figure 5) from the mouth of the flower (FA), the "neck" of the flower (FB), and the widest point of the flower (FC).

In addition to the measurements taken by Carroll (1996), measurements were taken of the length and width of each calyx lobe and of the internal floral characteristics. Inside measurements of the flower were taken by dissecting the flower along the longitudinal cross section and taking a thin (1 mm) slice off one side to expose the inner calyx. Morphological measurements were then taken of the stigma lobe length, the length of the anther openings, the height of reticulations, the extent the reticulations covered the calyx tube and the length of the trichomes inside the calyx (Figure 6).

The morphological differences between *A. contracta* and *A. rhombiformis* were analyzed using t-tests, non-parametric Wilcoxon tests, Discriminant Function Analysis (DFA) preceded by a Stepwise Discriminant Analysis (SDA), and Principle Component Analysis (PCA) in SAS (Swofford 2002). The Stepwise Discriminant Analysis builds a model of discrimination with one variable at a time to evaluate which variables contribute the most to the discrimination between groups. Once a variable is determined to help in the discrimination, it is added to the function. Morphological data collected for *A. contracta* and *A. rhombiformis* were analyzed in a Stepwise Discriminant Analysis to determine the most important characteristics for the separation of *A. contracta* and *A. rhombiformis* and then those variables that contributed to the discrimination were analyzed using DFA. The Discriminant Function Analysis builds a function to determine whether groups differ with regard to the mean of a variable and uses that variable to predict membership within the groups. The morphological characters were also used in the PCA to determine the structure of the variables. Analyses were performed with and without the addition of Carroll's (1996) data on *A. contracta* from Tennessee.

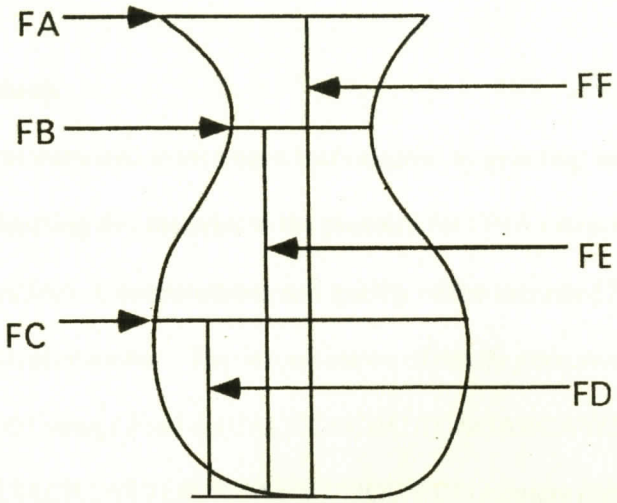

Figure 5: Schematic drawing of the measurements taken from each flower. FA measures the opening of the flower from calyx lobe to calyx lobe, FB measures the width of the calyx neck, FC measures the width of the widest point in the flower, FF measures the total length of the flower, FE measures the length from the neck to the base, and FD measures from the widest point to the base.

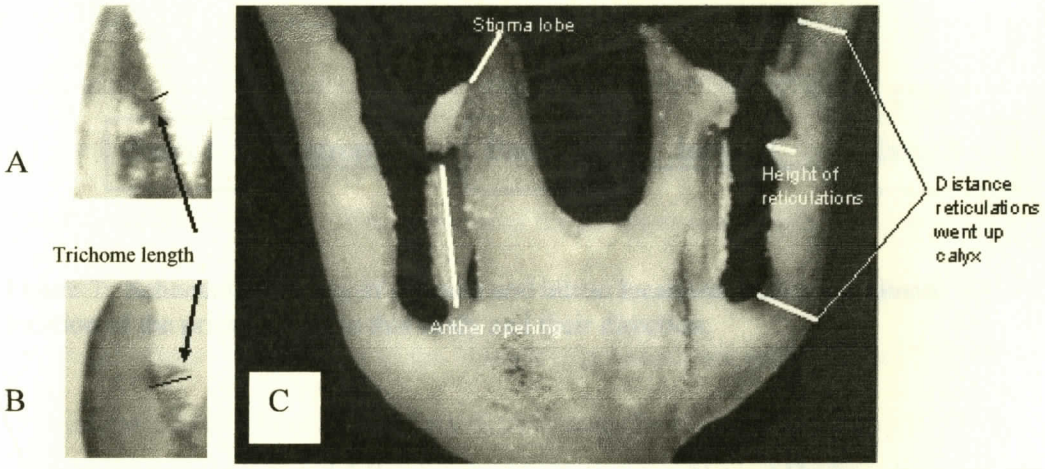

Figure 6: Inside measurements taken from each flower. A: Trichome length on the inside of the calyx of *A. rhombiformis*. B: Trichome length on the inside of the calyx of *A. contracta.* C: Photograph of 1 mm cross section used to expose inner details of the calyx including height of reticulations, stigma lobe length and length of anther opening.

Molecular Methods

DNA was extracted from frozen leaf material by grinding leaves to a fine powder using liquid nitrogen and subjecting this material to the protocol for DNA extraction by Qiagen DNeasy plant mini kits (Qiagen Inc). Concentrations and quality of the extracted DNA were analyzed using a NanoDrop spectrophotometer. The second intron of the *lfy* gene was then PCR amplified (GeneAmp PCR system 9700) using LFsx1-1 (CACCCACAC CCAGARCAYCCITTYATIGTIACGA) and LFtxr (CCTGCCIACRTARTGICKCAT YTTIGGYTT) primers (Frohlich and Meyerowitz 1997) (Figure 7) and GoTaq Green Master Mix (Promega). Reactions contained 12.5µl GoTaq, 10.5µl Nuclease free water, 0.5µl LFsx1-1 forward primer, 0.5µl LFtxr reverse primer and 1µl template DNA. PCR cycles consisted of 40 cycles of 95°C for 30 sec for DNA denaturation, 1 min of 48°C for primer annealing, 2 min at 72°C for primer extension, followed by 5 min of 72°C for completion of DNA synthesis.

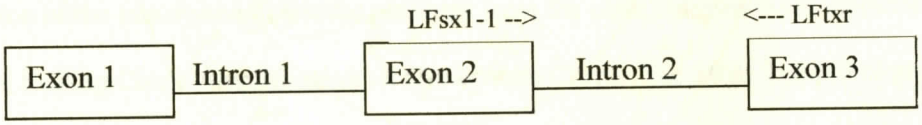

Figure 7: Schematic drawing of *lfy* exon and intron locations, with approximate location of the primers used in this study and their direction.

Samples were labeled following the North Carolina Natural Heritage Program 1996 record system. The H represents "*Hexastylis*" and C or R represents *contracta* or *rhombiformis* respectively and the last three numbers represent the population number, for example HC 004. The species and population is preceeded by the sequence letter and the number of the individual. Thus A, B, C, D, E, F, G or H represent the sequence while 1 or 2 represents the first or second individual from a

population. For example A1HC 004 represents sequence A from the first individual of *Asarum contracta* from population 004.

To better determine the relationship of *A. contracta* and *A. rhombiformis* to other species within the "*Hexastylis* clade" of *Asarum*, one individual from the remaining 10 "*Hexastylis*" species and one individual from each variety of *A. arifolia* (Michaux) Small were included in the DNA samples used in the analysis. Other taxa included in the analysis were one individual from *A. hatsushimae* Maekawa to represent the "*Heterotropa*" clade, and one individual from *A. canadense* L. and *Isotrema macrophylla* (Lamark) Reed.

All of the samples used in the analysis were purified after electrophoresis of the entire PCR product in 1% agarose gel using Qiagen Gel Extraction kits following the manufacturer's protocol (Qiagen Inc). The purified PCR products were then cloned using the Topo TA Cloning Kit (Invitrogen Inc) according to the manufacturer's protocol. Transformed cells were grown on plates containing Lauria Broth and ampicillin (Emily Gillespie, *personal communication*). Eight colonies per cloning reaction, per individual were selected for use in further protocols. After screening and proliferation of the transformed cells the plasmids from the eight colonies were amplified using TempliPhi Rolling Circle Amplification kit (Amersham Corp). The 10 µl TempliPhi product was diluted with 15 µl of ddH_2O. The presence of the *lfy* insert was verified by restricting 3 µl of the diluted TemliPhi product with the *Eco*RI restriction enzyme (Promega Corp) and checked on a 1.0% agarose gel. Once checked, five of the TempliPhi products that contained the *lfy* insert were sent to Life Sciences Core Laboratories at Cornell University for cycle sequencing using the Applied Biosystems Automated 3730 DNA Analyzer (Life Sciences Core Laboratories). Forward DNA sequences were analyzed and compared for two individuals from four populations of *A. contracta* and *A. rhombiformis,* one individual from the remaining "*Hexastylis*" species, and one individual each of *A. hatsushimae*, *A. canadense* and *Isotrema macrophylla.* Sequences were edited and aligned by eye using McClade (Maddison and Maddison 2005). Sequences were trimmed of vector sequences and

checked for similarity with sequences already deposited in public databases using BLAST from NCBI. Sequence data were analyzed using PAUP version 4.0 (Swofford 2002).

Phylogenetic analyses were inferred using maximum parsimony (MP) criteria in PAUP version 4.0 (Swofford 2002). A full heuristic search with additions automatically added was used to generate the tree based on maximum parsimony in PAUP. Gaps were encoded as missing data, and all characters were weighted equally. The consensus tree of 536,000 trees generated was used to assess relationships of 165 taxa with a 390 bp sequence. Consensus sequences were generated by combining the five sequences from each of the 31 individuals, leaving 31 sequences from the 165 acquisitions. The consensus sequences were then used to generate MP trees. The first three sequences of each individual were also used independently to generate MP trees. The four analyses using only 31 taxa were then compared to the full consensus tree for all 165 taxa in order to determine the best representation of the data set.

RESULTS

Ecological Results

Eight populations of *Asarum rhombiformis* and three populations of *A. contracta* were re-located in North Carolina from North Carolina Natural Heritage Program records (1996) and mapped using ArcMap GIS software (ESRI 2006) (Figure 8). The populations of *A. rhombiformis* tended to be to the southwest of the *A. contracta* populations with the exception of HC 006, which was located south and west of the HR 002 and HR New populations (Figure 8).

The drainages (NC OneMap 2006) that *A. contracta* and *A. rhombiformis* are found in are the French Broad River, the Broad River and the Little Tennessee River (Figure 9). The population of HR 006 was the only population located in the Little Tennessee River basin. The populations of HR 002, HR New and HC 006 appeared to be located on the edge of both the French Broad and the Broad River basins. The remaining populations were located in the French Broad River basin.

The location of *A. contracta* populations in Tennessee (Carroll 1996) were added to location data of *A. contracta* in North Carolina for comparison (Figure 10). The closest Tennessee population of *A. contracta* was located two counties away from the *A. contracta* populations in North Carolina, and may represent a population of *A. contracta* and *A. arifolia* intermediates.

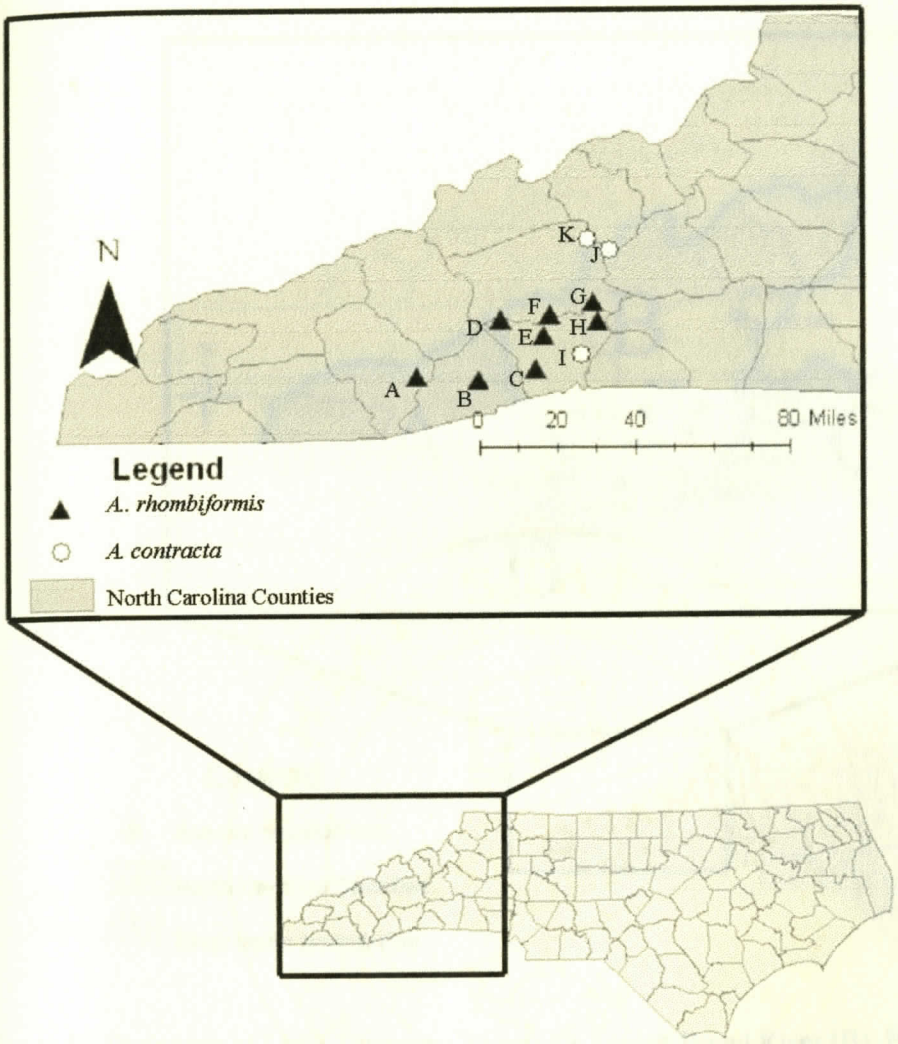

Figure 8: Location of *Asarum rhombiformis* and *A. contracta* sites used in this study. Data points collected using a Garmin GPS unit in 2006. The populations labeled A-H represent *A. rhombiformis* populations and I-K represent *A. contracta* populations. A = HR 006, B = HR 004, C = HR 008, D = HR 010, E = HR 001, F = HR 012, G = HR 002, H = HR New, I = HC 006, J = HC 003, K = HC 004.

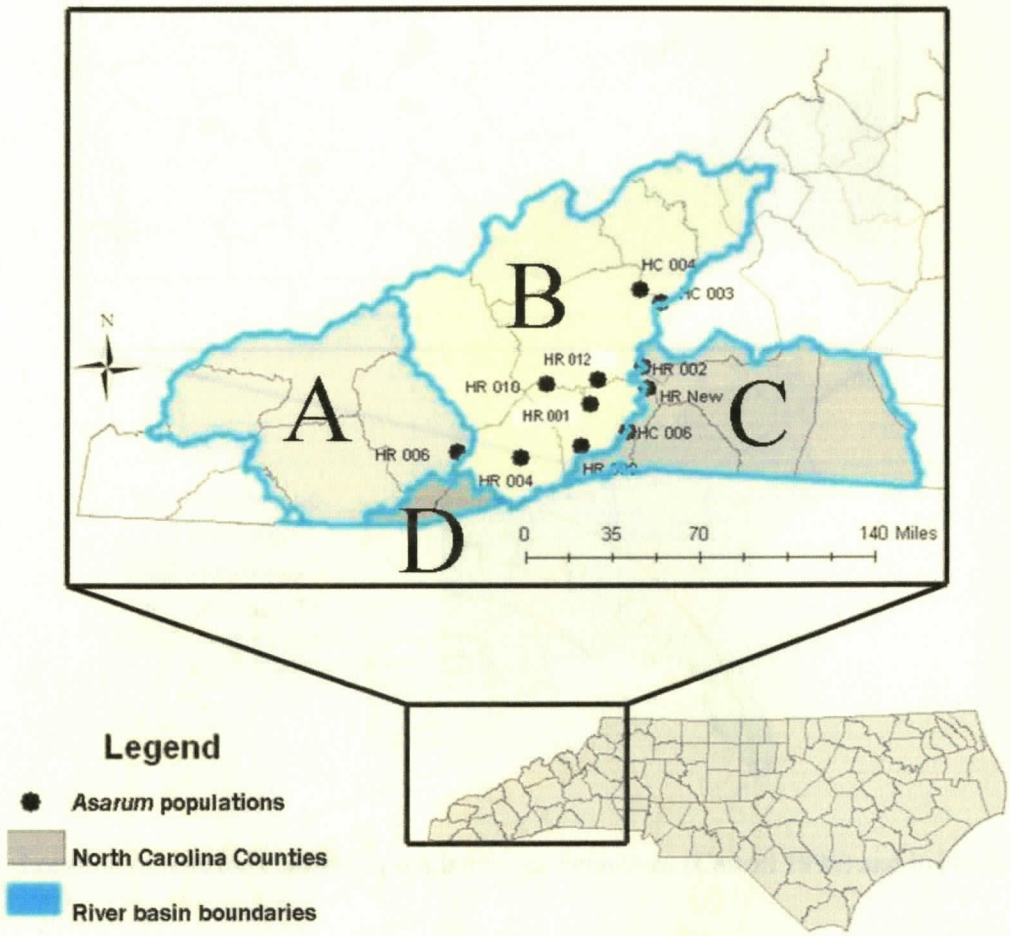

Figure 9: Drainages of Little Tennessee River (A), French Broad River (B), Broad River (C) and Savannah River (C) shown with locations of *A. rhombiformis* and *A. contracta* populations.

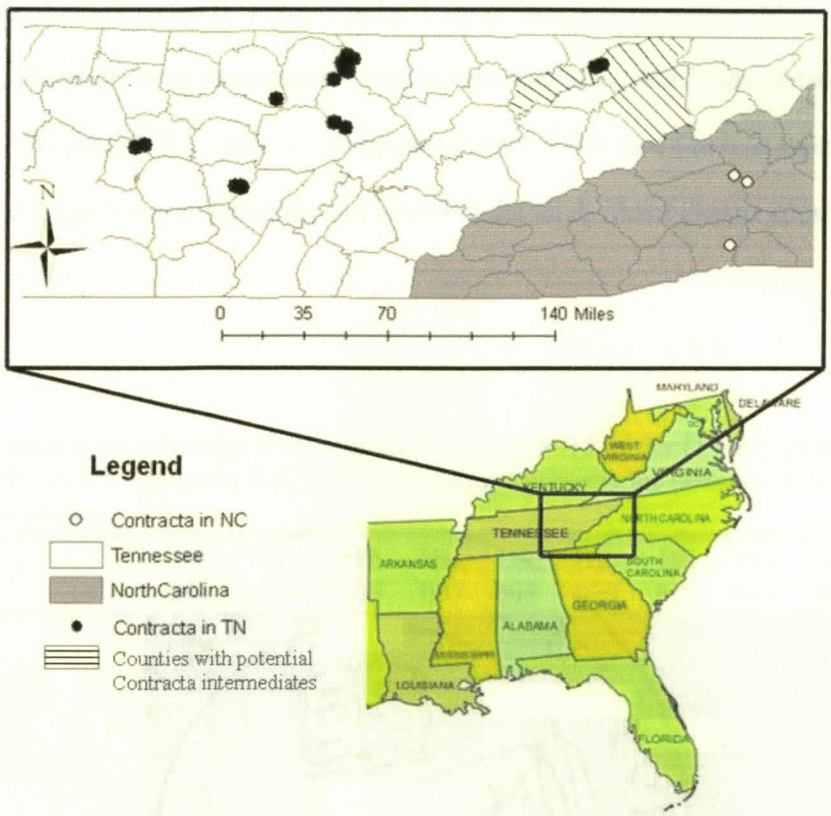

Figure 10: Location of *Asarum contracta* populations in Tennessee (Carroll 1996) and in North Carolina (2006).

The co-occurring vegetation was identified for all eight populations of *A. rhombiformis* and three populations of *A. contracta* in North Carolina using Newcomb (1977) and Weakley (2007; Appendix B). The eight populations of *A. rhombiformis* had a mean of approximately 39 co-occurring plant species, while the three populations of *A. contracta* had a mean of approximately 37 co-occurring plant species. The number of co-occurring plant species had a non-significant *p* value of 0.30 when analyzed with a t-test. From the *A. rhombiformis* populations there were 48 plant species found exclusively in one or more *A. rhombiformis* populations. There were only 10 plant species found exclusively in *A. contracta* populations. The most common species found to co-occur with *A. contracta* and *A. rhombiformis* were *Acer rubrum* L., *Galax urceolata* (Poiret) Brummitt, *Goodyera pubescens* (Willdenow) R. Brown, *Kalmia latifolia* L., *Liriodendron tulipifera* L., *Nyssa sylvatica*

Marshall, *Oxydendrum arboreum* (L.) de Candolle, *Polystichum acrostichoides* (Michaux) Schott, *Quercus alba* L., *Rhododendron maximum* L., *Smilax rotundifolia* L., and *Tsuga canadensis* L (Newcomb 1977, Weakley 2007; Table 1). *Magnolia fraseri* Walter and *Quercus rubra* L. were found in all of the *A. contracta* populations and some but not all *A. rhombiformis* populations (Appendix B).

Table 1: Most common co-occurring plant species present in 9 -11 of the 11 study populations of *A. rhombiformis* (HR) and *A. contracta* (HC). Black shading represents the occurrence of that plant species in that population.

Scientific name	HR 001	HR 004	HR 006	HR 007	HR 008	HR 010	HR 012	HR New	HC 003	HC 004	HC 006
Acer rubrum	■	■		■	■	■	■	■	■	■	■
Gaylax aphylla	■	■	■	■		■	■	■	■	■	■
Goodyera pubescens	■	■	■	■			■	■	■	■	■
Kalmia latifolia	■	■	■	■	■	■		■	■	■	■
Liriodendron tulipifera	■	■	■	■	■	■	■	■	■	■	■
Nyssa sylvatica	■	■			■	■	■	■	■	■	■
Oxydendrum arboretum	■	■	■	■	■	■	■	■	■	■	
Quercus alba	■	■		■	■	■	■	■	■	■	■
Polystichum acrostichoides	■	■	■	■			■	■	■	■	■
Rhododendron maximum	■	■	■	■	■	■		■	■	■	
Smilax rotundifolia	■	■	■	■		■	■	■	■	■	■
Tsuga canadensis	■	■	■	■		■	■	■		■	■

Using the Sorenson's Index of Community Similarity this study found that, *A. rhombiformis* and *A. contracta* populations were 67% similar in species. Individual populations of *A. rhombiformis* varied from being 36% -69% similar. The least similar *A. rhombiformis* populations were HR 006 and HR 012 while the most similar populations were HR 001 and HR 008. The *A. contracta* populations ranged in similarity from 48% - 49% similar in co-occurring species (Table 2). The populations of *A. contracta* that were the least similar were HC 003 and HC 006 while the most similar populations were HC 004 and HC 003. The individual populations of *A. rhombiformis* and *A.*

contracta ranged in similarity from HR 008 and HC 006 with 28% similarity, to HR 004 and HC 004 with 59% similarity.

The elevation data gathered by a handheld Garmin GPS unit for the all of the populations used in this study ranged from 472 meters to 845 meters (Table 3). The t-test comparing the mean elevation data for *A. contracta* and *A. rhombiformis* had a *p* value of 0.614, which showed that *A. contracta* and *A. rhombiformis* did not differ significantly in elevation.

Table 2: Sorenson's Index of Community Similarity for all of the plots

Plots	HR 001	HR 004	HR 006	HR 007	HR 008	HR 010	HR 012	HR New	HC 003	HC 004
HR 001	---									
HR 004	0.58	---								
HR 006	0.48	0.56	---							
HR 007	0.55	0.64	0.60	---						
HR 008	0.69	0.48	0.41	0.44	---					
HR 010	0.63	0.51	0.43	0.47	0.59	---				
HR 012	0.48	0.43	0.36	0.37	0.38	0.44	---			
HR New	0.59	0.49	0.51	0.46	0.53	0.55	0.59	---		
HC 003	0.55	0.45	0.42	0.47	0.41	0.52	0.53	0.56	---	
HC 004	0.51	0.59	0.46	0.52	0.40	0.42	0.56	0.47	0.49	---
HC 006	0.36	0.46	0.44	0.42	0.28	0.39	0.53	0.49	0.48	0.49

Table 3: Elevation data for *Asarum contracta* (HC) and *A. rhombiformis* (HR) populations.

Populations	Elevation in meters
HR 001	631
HR 004	697
HR 006	845
HR 007	748
HR 008	629
HR 010	630
HR 012	566
HR New	471
HC 003	700
HC 004	706
HC 006	557

Morphological Results

Morphological measurements were obtained from 28 *A. rhombiformis* flowers and 10 *A. contracta* flowers (Appendix C). The t-tests generated from SAS showed that the outside measurements of FC, FD, FE, and FF, were significantly different (Table 3). All five of the inside measurements were found to be significant according to the t-tests, or the non-parametric Wilcoxon test used for those measurements that were not normally distributed (Table 4).

Table 4: Results of t-test and Wilcoxon tests for the outside and inside floral characteristics. All measurements in millimeters.

Variable	DF	t Value	Pr > |t|
Outside Characteristics			
FA	11	0.65	0.5323
FB	14	2.04	0.0614
FC	15	5.20	0.0001*
FD	14	3.78	0.0020*
FE	24	4.43	0.0002*
FF	18	2.10	0.0503*
FLL◊	12	-----	0.0909
FLW	12	-1.02	0.3300
Inside Characteristics			
Height of reticulations	11	10.70	<0.0001*
Distance of reticulations	15	6.07	<0.0001*
Trichome length◊	11	-----	0.0003*
Stigma lobe length	9	2.51	0.0320*
Anther opening length◊	11	-----	0.0222*

◊ not normally distributed
* significant at $p < 0.05$

The Stepwise Discriminant Analyses (SDA) showed different variables were useful in characterizing the different species depending on the data analyzed. When only the outside measurements were used in the SDA, FB, FC, FD, and FLW were found to be the variables that were useful in separating *A. contracta* from *A. rhombiformis.* The DFA performed using FB, FC, FD, and

FLW (Figure 11) classified species 2 as *A. contracta* 90% of the time and species 1 as *A. rhombiformis* 93% of the time (Table 5).

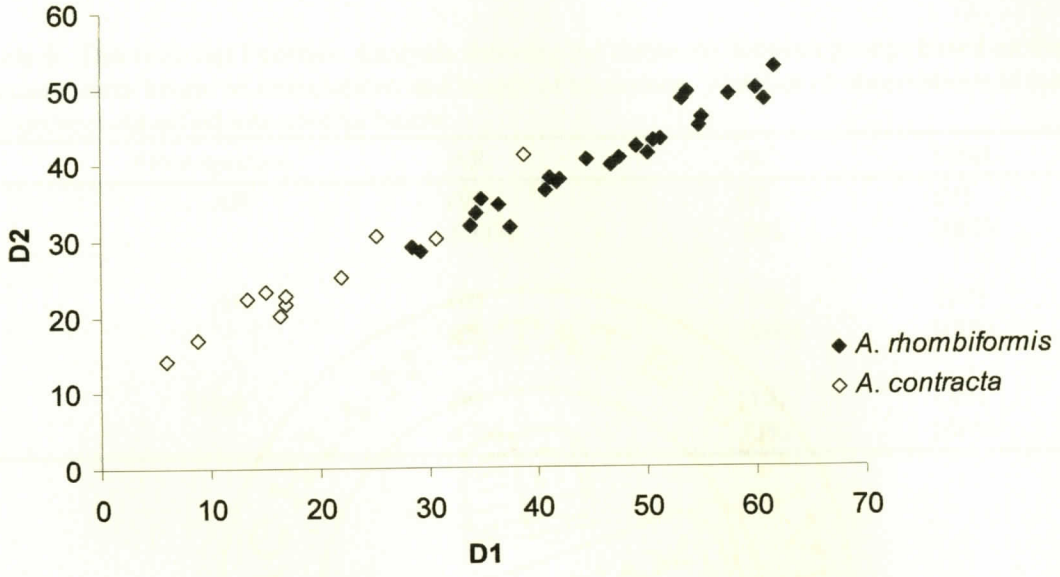

Figure 11: Graph of the DFA functions with FB, FC, FD and FLW used to separate the groups.

Table 5: Discriminant Function Analysis showing the distances between groups based on only outside characters FB, FC, FD and FLW. Number of observations in parenthesis and percent classified into species below.

From species	AR	AC	Total
AR	(26) 93%	(2) 7%	(28) 100%
AC	(1) 10%	(9) 90%	(10) 100%
Total	(27) 71%	(11) 29%	(38) 100%

When the inside measurements were used in a SDA, the height of the reticulations and the trichome length were found to be important classification criteria for the two species. Using those

two variables, the DFA (Figure 12) resulted in classifying species 2 as *A. contracta* 100% of the time and species 1 as *A. rhombiformis* 100% of the time (Table 6).

Table 6: Discriminant Function Analysis showing the distances between groups based on the inside measurements height of reticulations and length of trichomes. Number of observations in parenthesis and percent classified into species below.

From species	AR	AC	Total
AR	(9) 100%	(0) 0%	(9) 100%
AC	(0) 0%	(10) 100%	(10) 100%
Total	(9) 47%	(10) 53%	(19) 100%

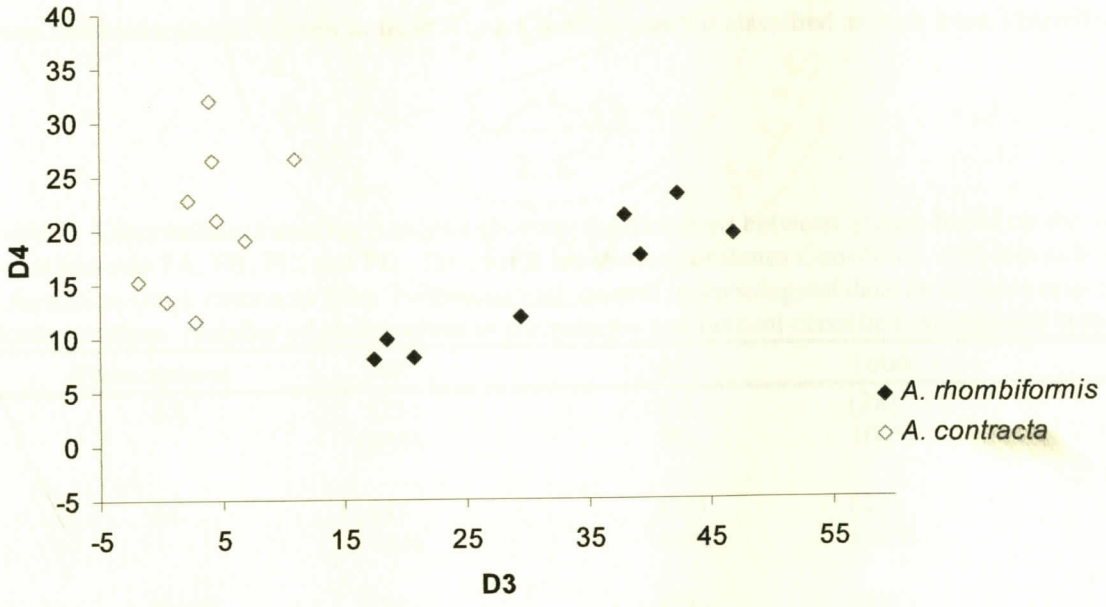

Figure 12: Using the height of reticulations and length of trichomes the DFA created functions that separate *A. rhombiformis* from *A. contracta* 100%.

When both the inside and outside measurements were entered into the SDA, height of reticulations and trichome length were found to be the values contributing to the classification of the species (Figure 12 and Table 6). Thus the SDA demonstrated no benefit to adding any of the outside measurements to classify *A. contracta* and *A. rhombiformis* as different species.

A DFA was performed using variables FA, FB, FC, FD, FF, to determine how individuals of *A. contracta* from Tennessee (Carroll 1996) and *A. contracta* from North Carolina would be classified when treated as one species (Table 7). The result was that the percent of *A. contracta* that matched *A. contracta* went from 90% when only North Carolina data were included down to 84% when combined with Tennessee data. When a DFA was performed using variables FA, FB, FC, FD, FF, to determine how individuals from *A. contracta* from Tennessee would fall out when treated as a different species of *A. contracta* from North Carolina, *A. contracta* from Tennessee was classified as unique 74% of the time (Figure 13, Table 8). When Tennessee *A. contracta* was added to the analysis and considered as a different species, *A rhombiformis* was classified 7% of the time to be *A. contracta* from Tennessee and *A. contracta* from North Carolina was not classified as such from Tennessee.

Table 7: Discriminant Function Analysis showing the distances between groups based on the outside measurements FA, FB, FC, and FD. Data for *A. contracta* combines Carroll's (1996) morphological information for *A. contracta* from Tennessee with current morphological data for *A. contracta* from North Carolina. Number of observations in parentheses and percent classified into species below.

From species	AR	AC	Total
AR	(23) 93%	(3) 7%	(28) 100%
AC	(4) 16%	(21) 84%	(25) 100%
Total	(30) 57%	(23) 43%	(53) 100%

A Principle Component Analysis (PCA) was performed combining morphological data for *A. contracta* and *A. rhombiformis* from North Carolina collected in this study with Carroll's (1996) morphological information for *A. contracta* from Tennessee. The first principle component described 52% of the variation while the second principle component accounted for 21% of the variation (Table 9). The eigenvalues of the PCA were 3.091 for the first principle component and 1.258 for the second component and together they accounted for 90.7% of the total variance. The graph of principle component one versus principle component 2 (Figure 14) showed that there was no clear separation between *A. contracta* from Tennessee and North Carolina or *A. rhombiformis* from North Carolina.

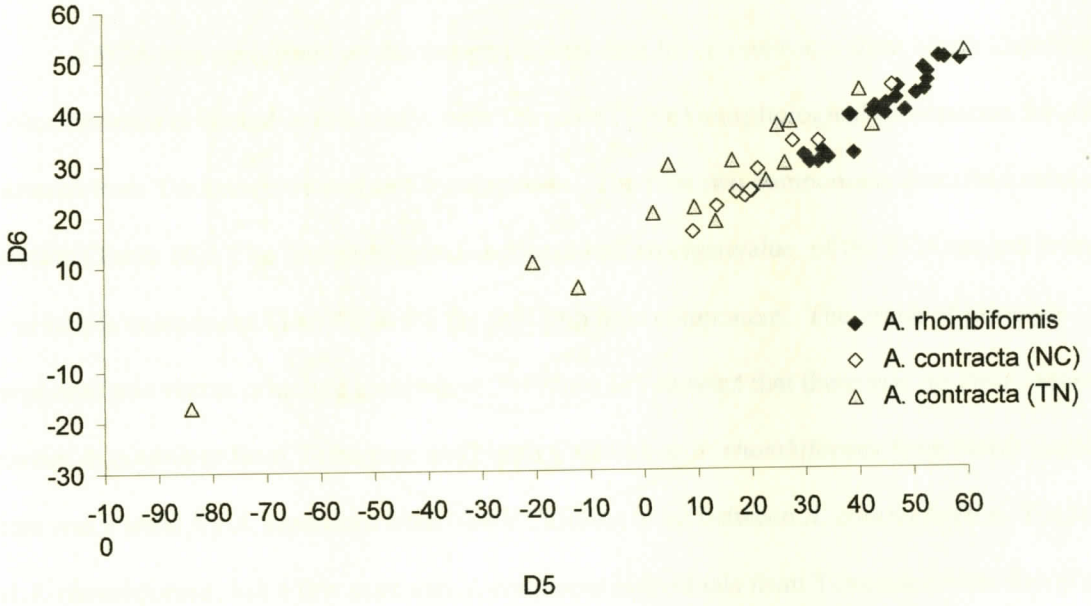

Figure 13: Discriminant Function Analysis using variables FA, FB, FC, FD, FF, to determine what the distance of separation was between *Asarum contracta* from North Carolina and Tennessee and *A. rhombiformis*.

Table 8: Discriminant Function Analysis showing the distances between groups based on the outside measurement FA, FB, FC, and FD. Data for *Asarum contracta* separated Carroll's (1996) morphological information for *A. contracta* from Tennessee (TN) and current morphological data for *A. contracta* from North Carolina (NC). Number of observations (in parentheses) and percent classified into species (below).

From species	AR	AC (NC)	AC (TN)	Total
AR	(23) 82%	(3) 11%	(2) 7%	(28) 100%
AC (NC)	(1) 10%	(9) 90%	(0) 0%	(25) 100%
AC (TN)	(2) 13%	(2) 13%	(11) 74%	(15) 100%
Total	(26) 49%	(14) 26%	(13) 25%	(53) 100%

A PCA was performed on the morphological data for *A. contracta* from North Carolina and *A. rhombiformis* collected in this study, with Carroll's (1996) morphological information for *Asarum contracta* from Tennessee treated as a third species. The first two components described most of the variation (Table 10). The first principle component had an eigenvalue of the PCA ranged from 0.002 for principle component 11 to 4.526 for the first principle component. The graph of principle component one versus principle component 2 (Figure 15) showed that there was no clear separation between *A. contracta* from Tennessee and North Carolina or *A. rhombiformis* from North Carolina. There was a trend for *A. contracta* from North Carolina to be between *A. contracta* from Tennessee and *A. rhombiformis*, but a few scattered *A. contracta* individuals from Tennessee were found among the *A. rhombiformis* individuals.

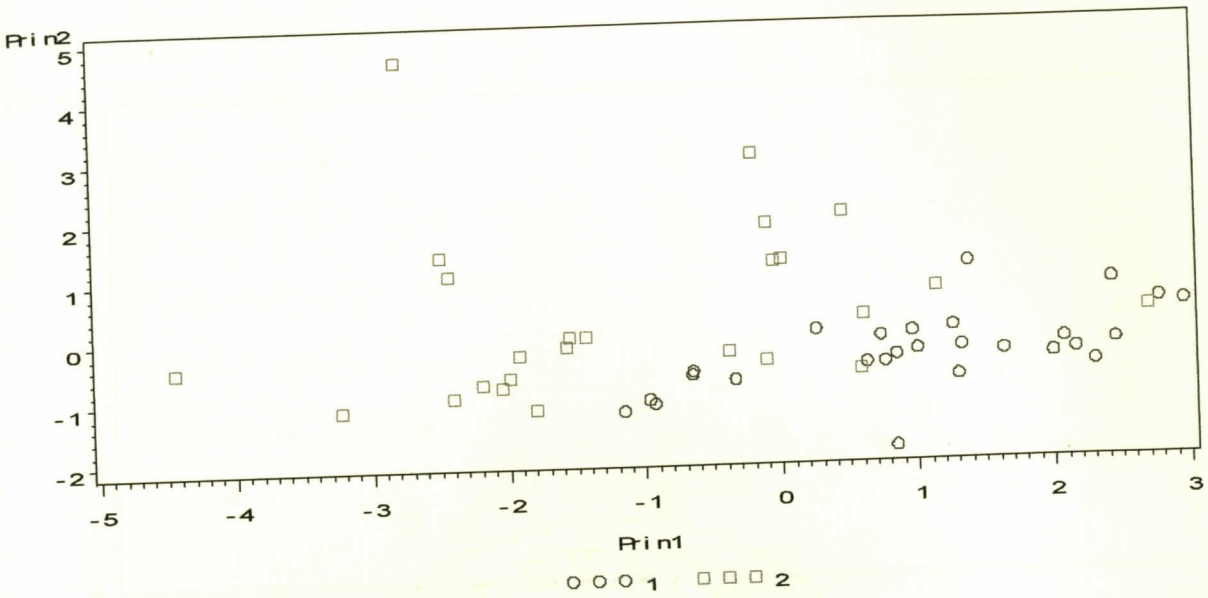

Figure 14: Principle components analysis with *Asarum contracta* from Tennessee and North Carolina represented by species 2 and species 1 represented by *A. rhombiformis.*

Table 9: Results from the PCA with *Asarum rhombiformis* and *A. contracta* from Tennessee and North Carolina as one species. Only the variables with eigenvalues greater than 0.5 shown.

PCA variable	Eigenvalue	Difference	Proportion of variance	Cumulative
1	3.091	1.832	80.515	0.515
2	1.259	0.717	10.210	0.725
3	0.542	0.040	00.090	0.815
4	0.502	0.150	00.084	0.899

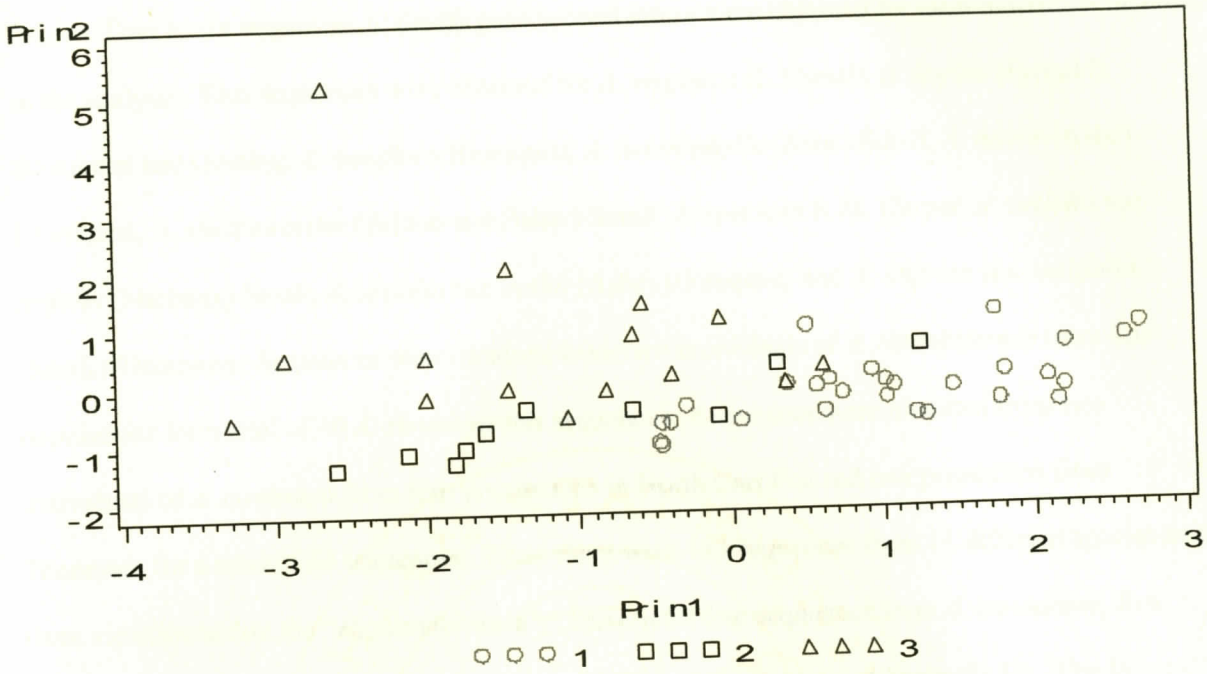

Figure 15: Results of PCA with *Asarum rhombiformis* and *A. contracta* from Tennessee and North Carolina as different species. Species 1 is *A. rhombiformis*, species 2 is *A. contracta* from North Carolina and species 3 is *A. contracta* from Tennessee.

Table 10: Results from PCA with *Asarum rhombiformis* and *A. contracta* from Tennessee and North Carolina as different species. Only the variables with eigenvalues greater than 0.5 shown.

PCA variable	Eigenvalue	Difference	Proportion	Cumulative
1	3.091	1.832	80.515	0.515
2	1.259	0.717	10.210	0.725
3	0.542	0.040	00.090	0.815
4	0.502	0.150	00.084	0.899

Molecular Results

Four to six sequences of the *lfy* gene second intron were obtained for each individual included in the analysis. Five sequences were obtained for *A. virginica* (L.) Small, *A. lewisii* (Fernald) Blomquist and Oosting, *A. naniflora* Blomquist, *A. heterophylla* (Ashe) Small, *A. minor* (Ashe) Blomquist, *A. shuttleworthii* (Britten and Baker) Small, *A. speciosa* R.M. Harper, *A. arifolia* var. *arifolia* (Michaux) Small, *A. arifolia* var. *ruthii* (Ashe) Blomquist, and *A. arifolia* var. *callifolia* (Small) Blomquist. Sequences were obtained from two individuals of *A. rhombiformis* from four populations for a total of 40 *A. rhombiformis* sequences. Sequences were obtained from two individuals of *A. contracta* from four populations in North Carolina and one population from Tennessee for a total of 53 sequences. Thus, there were 143 sequences from 11 different species and three varieties within the "*Hexastylis* clade". There were five sequences from *A. canadense*, five from the *Isotrema macrophylla* and six sequences from *A. hatsushimae* (Appendix D). The *lfy* sequences ranged from 347-379 bp in length (Table 9). The number of constant characters ranged from 348 in *A. hatsushimae* to 379 in *A. canadense*. Individuals from the "*Hexastylis* clade" had 20 parsimony informative characters compared to zero parsimony informative characters found from the five *Isotrema macrophylla* sequences.

Table 11: Sequence characteristics of the *lfy* gene 2nd intron region with 82 bp from the 2nd 3 exon, for 165 accessions of Aristolochiaceae.

Sequence characteristic	"*Hexastylis*"	"*Heterotropa*"	*Asarum*	*Isotrema*
No. accessions	143	6	5	5
Length variation	347-353bp	347-348bp	378-379bp	357-358bp
No. aligned positions	353	348	379	358
No. positions constant	291	329	371	291
No. variable not informative	42	15	7	9
No. positions parsimony informative	20	4	1	0

The "*Hexastylis* clade" had a 31 bp gap when aligned with *A. canadense*. The six sequences from the *A. hatsushimae* shared the 31 bp gap with the "*Hexastylis* clade". Therefore, the individuals

representing the *Asiasarum* + *Hexastylis* + *Heterotropa* clade shared a 31 bp indel. When aligned against the *Asarum* genus the five *Isotrema* Rafinesque sequences had 13 indels ranging from 1 bp to 8 bp in length.

The consensus tree of 536,000 trees from the full heuristic search of 165 taxa with a 390 bp sequence, showed no resolution for any relationships within the "*Hexastylis* clade". The relationships with boot strap support were the five acquisitions from *Isotrema* with 100% bootstrap support and the five accessions from *A. hatsushimae* with 81% bootstrap support. Consensus sequences for the 31 individuals were generated from the 165 acquisitions and again no structure was found for relationships within those 31 individuals with a heuristic search. The bootstrap analysis on those 31 consensus sequences, as well as the 18 consensus sequences for *A. contracta* and *A. rhombiformis,* showed a bootstrap value of 65 for the relationship of HC 006 individual 1 and HC TN individual 2 (Figure 16). The first three sequences of each individual were also used independently to generate MP trees. The first and the third sequences showed similar structure, but the weak structure that was found was not supported by bootstrap values.

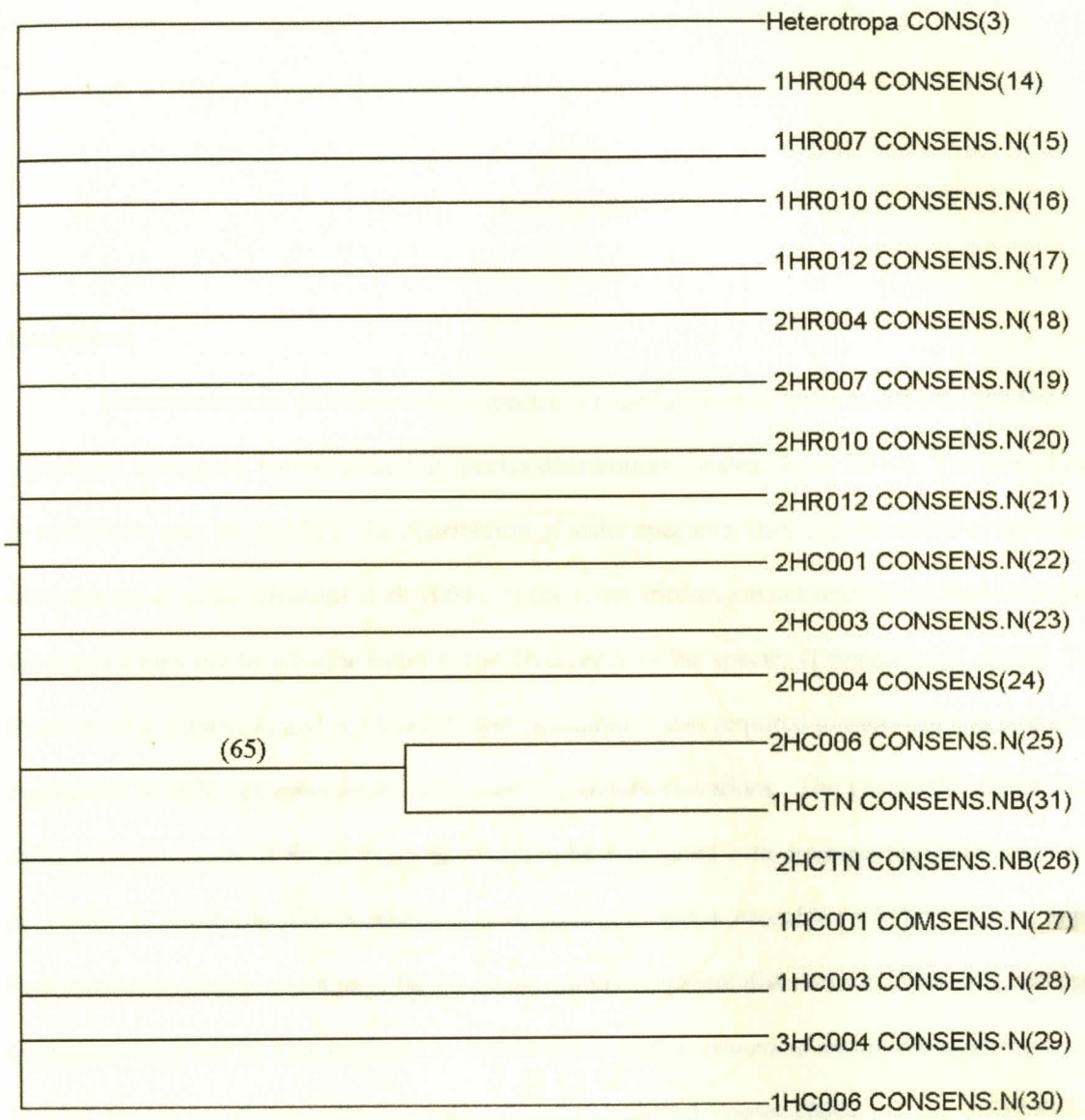

Figure 16: Bootstrap 50% majority-rule consensus tree for the consensus sequences of *A. contracta* and *A. rhombiformis* with *A. hatsushimae* as an outgroup.

DISCUSSION

Ecological

Determining the distribution of a species is a useful starting point to determining the significant ecological factors effecting species distribution (Graham et al. 2004). The role of ecology in speciation may be visible in the distribution of sister species if they consistently segregate in environmental space (Graham et al. 2004). If the sister species are not segregated then ecological divergence may not be a major factor in the divergence of the species (Peterson et al. 1999). The re-location of *A. contracta* and *A. rhombiformis* populations was required to establish whether they were segregated in different watersheds, or located at different elevations. The geographical distribution of different sublineages of *Ellipsoptera* appeared to be associated with drainage basins (Barraclough et al. 1998). In North Carolina populations of *A. contracta* and *A. rhombiformis* there did not appear to be a separation based on drainage basins or overall geographical distribution. Therefore, ecological divergence may not be a major factor in the divergence of *A. contracta* and *A. rhombiformis*, although species' ranges can change after speciation (Barraclough and Vogler 2000).

Elevation affects temperature, and often rainfall (Graham et al 2004) so determining the elevation of *A. contracta* and *A. rhombiformis* populations provided the first piece of information about potential differences in climactic requirements between taxa. Physiology, species richness and species ranges have been shown to be influenced by elevation gradients implying that elevation may be important in ecological species boundaries (Chown and Gaston 1999, Pither 2003, Naniwadekar and Vasudevan 2007). There was not a significant difference between elevation data for *A. contracta* and *A. rhombiformis* thus, elevation, does not appear to be a factor in the divergence of *A. contracta* and *A. rhombiformis.*

Studies have shown how species exhibit niche partitioning when growing in close proximity to other similar herbs (Graham et al. 2004, Queenborough et al. 2007). The Sorenson's Index of Community Similarity and the t-tests performed in this study did not show that *A. contracta* and *A. rhombiformis* exhibited niche partitioning. The ecological parameters of co-occurring plant species did not appear to exclude *A. contracta* and *A. rhombiformis* from growing in the same habitats in southwestern North Carolina. However, microhabitats were not examined and the soil characteristics which Padgett (2004) found to be a good indicator for the niche partitioning of the Heterophylla species complex within *Asarum* was not measured here and should be examined in the future.

Morphological

The vast number of morphological characters available and the potential that they are based on unlinked genes allow morphological characters to be more informative than molecular characters in certain phylogenetic situations (Jorgensen 2002, Wiens 2004, Smith and Turner 2005). In phylogenetic analyses that are dealing with rapid radiation, morphology is better able to reconstruct the proper clades due to the lag time in phylogenetic informative molecular characters (Jorgensen 2002, Smith and Turner 2005). Therefore, this study used morphological characteristics to ensure that if *A. contracta* from *A. rhombiformis* represented a case of rapid or recent radiation the divergence would be shown. The morphological characteristics were able to show significant differences between *A. rhombiformis*as and *A. contracta* while the second intron of *lfy* gene was unable to support differences suggesting that *A. contracta* and *A. rhombiformis*as may represent a case of recent speciation with insufficient time for molecular characters to diverge.

Morphological characteristics were also used in this study to resolve the conflicting conclusions between Gaddy (1986) and Barringer (1993) on the validity of *A. rhombiformis* as a distinct species separate from *A. contracta* based on morphology. In this study the inside morphological characteristics of height of reticulations and trichome length separated *A. contracta*

from *A. rhombiformis* 100% of the time. Due to the threatened status of *A. rhombiformis* and the endangered status of *A. contracta* in North Carolina it was important to know if the outside measurements alone would separate the two species so that removal of flowers was not required to identify the species. The outside morphological characteristics of FC and FB along with the ratios of FD and FLW were able to characterize *A. contracta* as *A. contracta* 90% of the time and *A. rhombiformis* as *A. rhombiformis* 93% of the time. In other studies exploring species boundaries, the discriminant function analysis was able to differentiate between species and was consistent with the molecular results (Green and Pustowka 1997, Fukami et al. 2004). In this study the floral characteristics were able to differentiate between *A. contracta* and *A. rhombiformis* although the results were not consistent with the molecular results. When investigating the relationships within subfamily Aristolochioideae, Wanke et al. (2006) also found conflicting support between morphological and molecular characters. The morphological results in this study supported the conclusion by Gaddy (1986) while the molecular results were inconclusive.

When North Carolina data gathered in this study for *A. contracta* and *A. rhombiformis* were added to Tennessee data for *A. contracta* gathered by Carroll (1996), the separation of the two species based on morphology was not as distinct. The DFA showed that when Tennessee *A. contracta* was added to the analysis, *A. contracta* classified as *A. contracta* went from 90% down to 84%, while *A. rhombiformis* continued to be classified as *A. rhombiformis* 93% of the time. *Asarum rhombiformis* was classified 7% of the time as *A. contracta* from Tennessee while *A. contracta* from North Carolina was not classified as *A. contracta* from Tennessee. There was also slight overlap between *A. contracta* from Tennessee and North Carolina and *A. rhombiformis* in the PCA. The connection between in *A. contracta* from Tennessee and *A. rhombiformis* brings up the question of whether the Tennessee species of *A. contracta* should be considered a different species or whether there is incomplete speciation taking place. The geographical features of the Appalachian mountains may be causing allopatric speciation as the mountains split *A. contracta*'s range into two isolated ranges

(Barraclough et al. 1998, Barraclough and Vogler 2000, Hewitt 2001). Thus, the Tennessee and North Carolina populations of *A. contracta* may be an example of speciation taking place.

Another possibility is that due to the gradient of the outside morphological characters and the subjective nature of taking character measurements (Stevens 1991, Gift and Stevens 1997, Scotland et al. 2003, Smith and Turner 2005). Carroll (1996) may have taken the measurements differently than they were taken in this study and thus there were slight morphological differences seen between *A contracta* from Tennessee and *A. contracta* from North Carolina. Despite the desire to only use discrete characters in phylogenetic analysis, at low taxonomic levels continuous characters may improve resolution (Stevens 1991, Boyd 2003). Therefore, the outside mophological characters were retained in this study. It could be argued that the outside morphological characters were continuous and thus there was difficulty in ensuring that the measurements taken in this study were identical to those taken by Carroll (1996).

Molecular

Preliminary results from *A. contracta* and *A. rhombiformis* showed two distinct copies of the *lfy* gene for each, which was interpreted to represent two alleles for a single locus due to only minor nucleotide differences without any major indels. Therefore, this study was originally designed to include *A. contracta* and *A. rhombiformis* with *A. canadense* and *Isotrema grandiflora* as outgroups was expanded to include one individual from each species within the "*Hexastylis* clade" and one individual from within the "*Heterotropa* clade" to determine if there was an obvious donor of the two alleles.

The expanded results showed a range of 4-5 distinct copies of the *lfy* gene among *A. naniflora, A. lewisii, A. heterotropa, A. minor, A. virginica, A. arifolia* var. *arifolia, A. arifolia* var. *ruthii, A. arifolia* var. *callifolia, A. contracta*, and *A. rhombiformis*. These multiple copies could represent both functional and non-functional copies of the gene (Archambault and Bruneau 2004,

Aagaard et al.2005) or they could represent alleles from multiple copies of a gene for a single locus (Smith and Baum 2006).

The short *lfy* sequence found by Archambault and Bruneau (2004) for many of the the taxa in Fabaceae had introns ranging from 85 bp to 235 bp in length which is comparible to the 206 bp intron found in the "*Hexastylis* clade" and "*Heterotropa* clade" of *Asarum* in my study. Therefore, if it is assumed that results of the short *lfy* in the Fabaceae are comparible to the short *lfy* found in the "*Hexastylis* clade", even though a long *lfy* sequence was absent, then some of the sequences found for the "*Hexastylis* clade" may represent pseudogenes or non-functional copies of the *lfy* gene. The short copy of *lfy* in Fabaceae was more problematic for phylogenetic inference of species relationships as it was found to be very incongruent with the chloroplast trnL intron as far as the relationships shown. Work with the chloroplast gene matK is underway for the "*Hexastylis* clade" but results are not yet available to compare with the results from the *lfy* gene (Bryan Neidenberger, *personal communication*).

My results showed areas in the exons that were variable with the second intron region being very short and lacking variability. Archambault and Bruneau (2004) found point mutations in the second exon, and the third exon had six indels varying in length from 2-12bp, while the second intron also varied in length. The variability found by Archambault and Bruneau (2004) in their multiple copies of the *lfy* gene were not found in this study, suggesting that the multiple copies in the "*Hexastylis* clade" are not as divergent as in Fabaceae, or that only one of the copies of *lfy* gene were successfully amplified.

The primers used in this study to amplify the second intron of *lfy* may not have amplified the other versions of the *lfy* gene in the "*Hexastylis* clade". The second intron primer set used by Aagaard et al. (2005) amplified two copies for two taxa but only one for the remaining taxa. In the subfamily Caesalpinioideae of the Fabaceae there were at least two distinct copies of the gene and the authors postulated that the species with only one copy of the *lfy* gene probably contained the alternate gene, but that it was not detected due to amplification error (Archambault and Bruneau 2004). The

degenerate nature of the primers was assumed to enable the LFsx1-1 and LFtxr primers to capture all of the possible *lfy* sequences (Frohlich and Meyerowitz 1997). However if there was an alternate *lfy* sequence that was less common or less available the primers may not have amplified the two *lfy* sequences equally (Archambault and Bruneau 2004, Aagaard et al. 2005).

The results for this study showed that different clones from the same individual had point mutations in the second intron of *lfy*. When compared to the electrophorogram the point mutations were not able to be edited due to the peak with the greatest intensity corresponding to the base call made. Those areas of point mutations occurred where there were double peaks with one clone having one peak higher than another clone at that same location. A previous study similarly found the sequences of the second intron of *lfy* to contain different nucleotides at two locations for two clones of the same individual and when they compared those results to the direct sequence they found overlapping peaks of equal intensity at each of the polymorphic sites (Oh and Potter 2003). Smith and Baum (2006) similarly found that most clones constituted minor sequence variants that were represented in the final matrix by consensus sequences. In fact, three of the 49 taxa contained two alleles that did not form a clade with others from the same accession (Smith and Baum 2006). Therefore, although possibly an amplification error, the point mutations not shared between clones of the same individual were not edited or removed from the analysis in this study.

Howarth and Baum (2005) were able to use the different alleles of the *lfy* gene along with two other introns from nuclear genes to create supported geneologies and speculate on hybrid origins of two species within *Scaevola* L. in the Hawaiian Islands. The Hawaiian Islands represent an environment that has temporal certainty and spacial simplicity which allows colonization and speciation to be determined with gene trees (Hewitt 2000), while the Southern Appalachians have temporal uncertainty and spacial complexity due to the refuges from the previous ice ages. The differences between the Hawaiian Islands and the Southern Appalachians may explain the differences in the efficacy of the *lfy* gene in phylogenetic studies performed here and by Howarth and Baum (2000).

The point mutations and the multiple *lfy* sequences were unable to resolve phylogentic relationships entirely on the *lfy* gene analysis performed here. The specific complexity of evolution of the whole nuclear genome makes it difficult and unwise to use a single nuclear region as a source of phylogenetic characters (Avise and Wallenburg 1997, Archambault and Bruneau 2004, Small 2004). With the resources available for this study only the one gene was possible at this time. Therefore, additional work with chloroplast and other nuclear genes should be conducted on the "*Hexastylis* clade" to ellucidate phylogenetic relationships.

Conclusions

The refuges provided by the Southern Appalachian Mountains during the last ice age may account for the numerous colonization locations of *A. rhombiformis* in western North Carolina and the disjunct distribution of *A. contracta*. The speciation of the ancestors within these different colonization locations could acount for the morphological differences seen (Negron-Oritz and Hickey 1996). The lack of concordance between the morphological and molecular information may be due in part to the faster rate of differentiation for morphological features and recent divergence (Negron-Oritz and Hickey 1996, Smith and Turner 2005). Another explanation is that the genetic isolation is caused by distance and, due to the small sampling area of western North Carolina, the genetic differences that separate *A. rhombiformis* and *A. contracta* as species or that support relationships in the "*Hexastylis* clade" of *Asarum* were not exposed (Friar et al. 2007). If the sampling area was too small that would explain the only relationship supported by bootstrap values was one sequence from an individual from *A. contracta* from North Carolina paired with a sequence from an individual from a Tennessee population of *A. contracta*.

While not strongly supported as distinct species, the imperiled nature of *A. contracta* and *A rhombiformis*, neccesitates that they continue to be recognized as distinct taxa until more data are obtained. Due to the inability to detect the number of *lfy* genes within the "*Hexatstylis* clade", the *lfy*

gene was not found to be phylogenetically informative at the species level. Therefore, other more appropriate genes should be identified for future studies with these species.

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APPENDIX A

Site descriptions based on information gathered in 2006 and 2007

HR 001- 2006 Found when looking for EO ID 18266
Directions: Polk CO, Saluda, NC - Take a left on SR 1122 and veer left onto SR 1191. Near the entrance to Jamboree Parking lot and between the parking lot and creek there is a telephone pole (DC60/ or DCGO). The population starts and goes further into the woods on the bank above the stream.
Threats: None obvious
Numbers: 32 leaves seen around the telephone pole and in the edge of the woods corresponding to about 13 individuals. The population advances into the woods but numbers were not taken.
GPS point in Decimal Degrees: N 35.46722 W 82.48194

HR 008- 2006 EO ID: Originally thought to be 14169 but population is on the west side of SR 1122.
Directions: Polk CO, Saluda, NC - Take a left on SR 1122 , veer right and continue on SR 1122 past Bald Eagle PVT Rd. On the right just before (50m before) 5025 and across from an old trailer on SR 1122 there is a small draw. The population lies there.
Threats: Development. The land abutting the population has been surveyed for Development/ subdivision.
Numbers: 76 leaves and about 50 individuals
GPS point in Decimal Degrees: N 35.26083 W 82.53306

HR 006- 2006-2007 found when looking for EO ID 14559
Directions: Transylvania CO, Rosman, NC - Turn left on SR 1326 off of Hwy 215. Cross the bridge and take a right onto SR 1379. The population is between the road and the river within 1/2mile of SR 1326 and SR 1379 junction.
Threats: There is a cess pool and evidence of beavers.
Numbers: 230 leaves belonging to about 100 individuals.
GPS point in Decimal Degrees: N 35.23083 W 82.97722

HR 007- 2006-2007 Found when looking for EO ID 7009
Directions: Transylvania CO, Rosman, NC - 4/5 mile south of SR 1326 on Hwy 215. Park on right side (N) of road before the guard rail and head down the slope to the river. The

population is located at the junction of the tributary and the French Broad River in the south corner. Not exactly EO ID 7009 because on the East side of the of the North Fork of the French Broad River but it is located 4/5 south of EO ID 14559 or # 006
Threats: none obvious
Number: 275 leaves for about 100 individuals spread across the hill side from the road to the creek.
GPS: no satellite reception

HR 010- 2006-2007 Found when looking for EO ID 2505
Directions: Henderson CO, Flat Rock, NC - Going south on Greenville Hwy 225 from Upward Rd go through two lights and 1.5 miles on the left is Red Fox lane. Travel across the bridge and the main population is on the right side of the road on the hill above the stream. There is another population if you continue down the stream but there were not as many individuals concentrated in one area so was not used in this study.
Threats: Development- In 2007 the bridge was being upgraded to facilitate the development of new roads and what appear to be lots between Greenville Hwy 225 and the stream.
Number: 43 leaves for 20 individuals in the main population. 35 leaves on the east side of the creek in a different section of the population.
GPS Point in Decimal Degrees: N 35.44777 W 82.66778

HR New- 2006
Directions: Polk CO, - At Saluda exit off of HWY 26 take a left and continue on Holbert Cove Rd. The population is on the west side of Holbert Cove road after crossing the first bridge and before 5445 Holbert Cove Rd. There is a parking area and an old logging road that parallels the river. Between the river and the logging road there are a few individuals but the ones used in this study are upslope of logging road.
Threats: none obvious
Numbers: 70 leaves (upslope of logging road) for about 30 - 50 individuals.
GPS Point in Decimal Degrees: N 35.44277 W 82.30306

HR 002 - 2006 Associated with EO ID 18138
Directions: Polk CO, - At Saluda exit off of HWY 26 take a left and continue on Holbert Cove Rd. The population is on the east side of Holbert Cove road before crossing the first bridge over the river and before 5445 Holbert Cove Rd. The two individuals measured were located on the north side of the river before crossing the river for the first time to go to Bradley Falls.
An extensive search was not conducted so numbers are unknown.
GPS Point in Decimal Degrees: N 35.51083 W 82.32417

HR 004- 2006-2007 Found when looking for EO ID 14914
Directions: Cedar Mountain, NC - Green River Rd on the boarder of NC and SC. Green River Preserve, Schenk Family land. Between the road and the river before Big Laurel creek.
Threats: Campers but no severe threats
Numbers: The population numbers will be split into three areas. One is close to a field and a Hemlock grove = 30 leaves, about 10 individuals. Further down the Bear trail by a fallen tree and old jagged stump along the river before the trail falls off = 180 leaves for about 100 individuals. Population on the hill by Big Laurel creek had about 56 leaves for about 20 individuals. Past Big Laurel Creek on the south side of the road between the river and the road by the fish feeder there were

40 leaves and about 10 individuals. All along the Green River Rd there are scattered individuals and small concentrated populations
GPS Point in Decimal Degrees: N 35.22083 W 82.74972

HR 012- 2006-2007 found when looking for EO ID 461
Directions: Henderson Co., East Flat Rock, NC - Going east on Upward rd, take a right on Big Hungry Rd (SR 1802). Stay on Big Hungry Rd (i.e. Turn left and then turn right to stay on Big Hungry Rd) until turning right onto Galimore Rd (SR 1956). Follow Galimore Rd to the base of the hill right before end of state maintenance. There is a parking lot for kayakers and two parking spots for hunters or fishermen. Park and walk down hunting trail (2007 road construction obscured the trail as a silt fence was put up across it and dirt piled up). As of 2006 and 2007 there was a fallen tree about 200m from Galimore Rd. At that tree the population started. There were a few scattered individuals upslope of the trail but the main population was located between the trail and the creek.
Threats: None obvious. There was one *Lonicera japonica* individual found in the population.
Numbers: 235 leaves for about 100 individuals.
GPS Point in Decimal Degrees: N 35.38889 W 82.50611

HC 006- 2006-2007 Found when looking for EO ID 7225
Directions: Henderson Co., East Flat Rock, NC - Going east on Upward rd, take a right on Big Hungry Rd (SR 1802). Stay on Big Hungry Rd (i.e. Turn left and then turn right to stay on Big Hungry Rd). Park at the bridge (only large cement bridge on SR 1802). Walk up Lower Hungry river on the left side (N) of the river. Cross two small trickles of a stream (small dips in the trail) 2007, the second trickle was a small ravine about 4 feet deep, in the trail. The population is located between the trail and the river just past the second trickle.
Threats: Beavers dragging trees down slope could uproot several individuals
Numbers: 275 leaves for about 100 individuals
GPS Point in Decimal Degrees: N 35.31083 W 82.36777

HC 003- 2006-2007 EO ID 1579
Directions: Buncombe Co., Black Mountain NC - Between 9-10 miles south on Hwy 9 from where HWY 40 crosses Hwy 9. The Havens is a new development on the left (east) side of Hwy 9 located right before Clear Branch Church. Clear Branch Church is located on the corner of Stroud Valley Rd (SR 2790) and Hwy 9. The population is all along the hillside above the stream on the east side of Hwy 9 across from Clear Branch Church.
Threats: Development
Numbers: 221 leaves for about 150 individuals. Most of the plants had small 1-2 leaves. Not many flowered (only about 5) in 2006. Found 10 of the most robust plants flowering in 2007.
GPS Point in Decimal Degrees: N 35.70444 W 82.26639

HC 004 - 2006-2007 Found when looking for EO ID 7629 but on the east side of SR 2797 1/2 mile from junction of SR2797 and NC 9, so possibly a subpopulation of EO ID 7629.
Directions: Buncombe Co., Black Mountain NC - Between 10.5 miles south on Hwy 9 from where HWY 40 crosses Hwy 9. Rock Creek Rd (SR 2797). Rock Creek Rd is a loop but if the 1st Rd on the left after Old Fort Rd is taken the population is 0.5 miles south of where SR 2797 turns off of Hwy 9 on the east side of the gravel road. The land owner may be Furnis. Population is on hill side above small stream.
Threats: development - the land just north of the population was logged and sold.

Numbers: 20 feet east of SR 2797 there is a concentration of 54 leaves and about 20 individuals. Head east from this lower population and there is another population near the top of a hill with 70 leaves and about 35 individuals.
GPS Point in Decimal Degrees: N 35.74194 W 82.34389

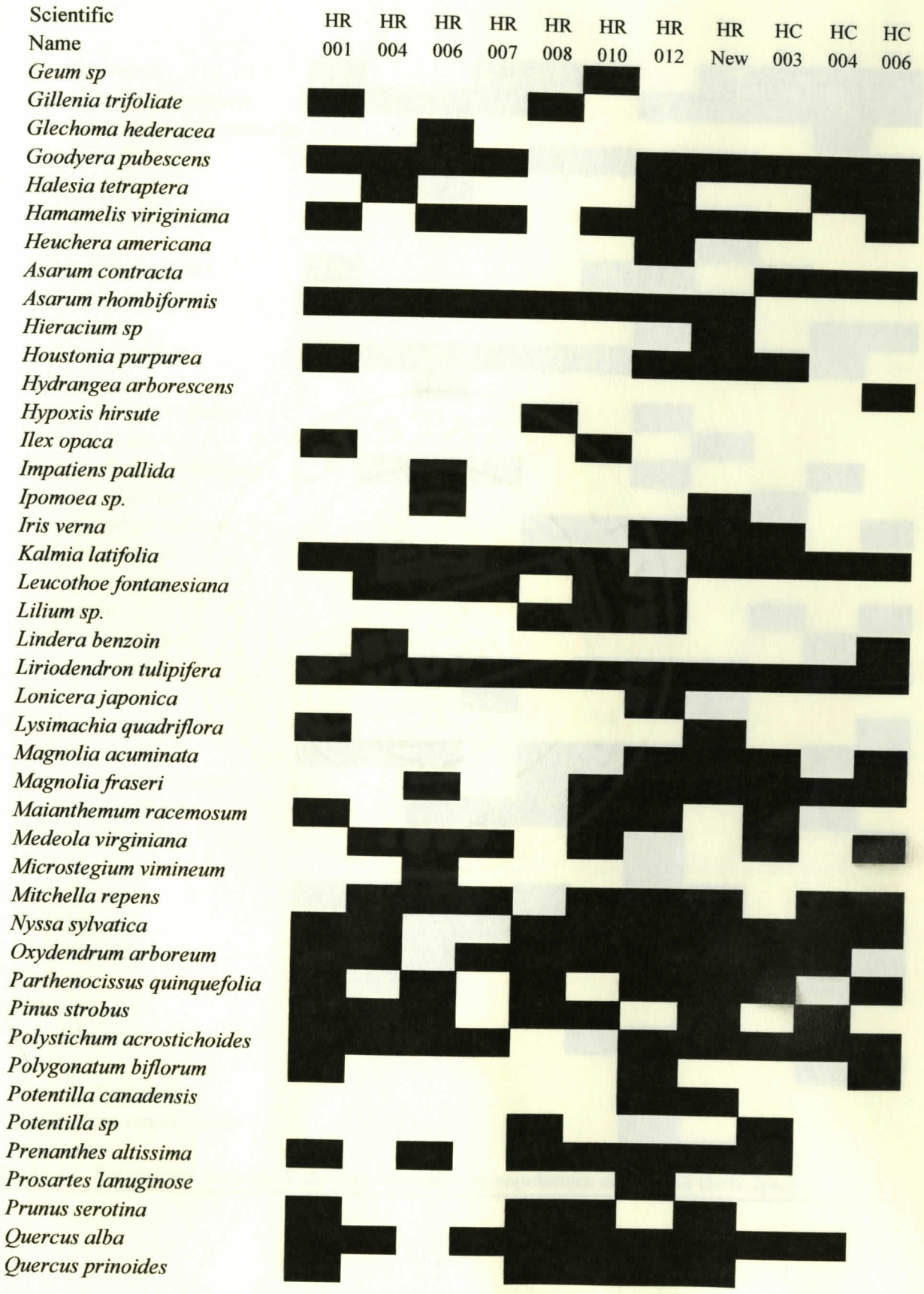
HC 001 - 2007
Directions: Henderson Co., Hendersonville, NC - From HWY 26 head east on Hwy 64. Turn right on Rockwood Dr. Travel 0.4miles up Rockwood Dr. Population is located at 121 Rockwood Dr. Hendersonville, NC 28792. Dr. Petillo collected *H. contracta* from both banks of Ostel's Pond in the 1950's. Now the population is located on the north facing slope above the stream. Ostel's pond has been drained and now a small stream winds through the boundary between homeowner's land. The population is up to and crossing the driveway.
Threats: English Ivy coming from yard is about 10cm from the first individual found in population.
Numbers: 75-100 individuals with about 20 plants flowering
GPS Points not available

APPENDIX B

Co-occurring plant species

List of co-occurring species and corresponding Hexastylis populations.

Scientific Name	HR 001	HR 004	HR 006	HR 007	HR 008	HR 010	HR 012	HR New	HC 003	HC 004	HC 006
Acer rubrum	■	■		■	■	■	■	■	■	■	■
Aesculus sp.			■				■	■	■		
Allium tricoccum										■	■
Amelanchier sp.			■								
Amphicarpaea bracteata							■	■			
Aplectrum hyemale										■	
Arisaema sp.							■	■	■	■	■
Arundinaria appalachiana				■							
Aster sp.							■	■		■	
Betula alleghaniensis							■	■	■	■	■
Betula lenta	■	■	■	■	■	■	■	■	■		
Calycanthus floridus						■	■	■	■	■	■
Cardamine diphylla			■								
Carex sp.							■		■		
Carpinus caroliniana										■	■
Carya sp.	■	■	■		■		■	■			
Chamaelirium luteum							■	■			
Chimaphila maculate	■	■		■	■	■	■	■	■	■	■
Clethra acuminate			■				■	■	■		
Conopholis americana							■	■	■		
Cornus alternifolia								■			
Cornus florida	■		■	■				■		■	
Dennstaedtia punctilobul	■					■					
Dioscorea villosa					■	■	■				
Epigaea repens										■	
Epipactis helleborine							■	■			
Euphorbia corollata					■						
Fagus grandifolia						■			■		
Galax urceolata	■	■	■	■	■	■	■	■	■	■	■
Gaylussacia baccata					■	■					


Scientific Name
HR 001
HR 004
HR 006
HR 007
HR 008
HR 010
HR 012
HR New
HC 003
HC 004
HC 006
Geum sp
Gillenia trifoliate
Glechoma hederacea
Goodyera pubescens
Halesia tetraptera
Hamamelis viriginiana
Heuchera americana
Asarum contracta
Asarum rhombiformis
Hieracium sp
Houstonia purpurea
Hydrangea arborescens
Hypoxis hirsute
Ilex opaca
Impatiens pallida
Ipomoea sp.
Iris verna
Kalmia latifolia
Leucothoe fontanesiana
Lilium sp.
Lindera benzoin
Liriodendron tulipifera
Lonicera japonica
Lysimachia quadriflora
Magnolia acuminata
Magnolia fraseri
Maianthemum racemosum
Medeola virginiana
Microstegium vimineum
Mitchella repens
Nyssa sylvatica
Oxydendrum arboreum
Parthenocissus quinquefolia
Pinus strobus
Polystichum acrostichoides
Polygonatum biflorum
Potentilla canadensis
Potentilla sp
Prenanthes altissima
Prosartes lanuginose
Prunus serotina
Quercus alba
Quercus prinoides

Footnote: Populations with black shading represent the populations containing those species.

APPENDIX C

Measurements of Floral Morphology

Mean +/- SE of outside morphological measurements in millimeters for *Asarum contracta* (HC)

Location	FA	FB	FC	FD	FE	FF	FLL	FLW
HC 003	6.3	7.1	10.7	5.7	12.6	19.5	3.4	5.1
HC 004	8.2	7.7	11.3	6.6	12.7	19.5	3.3	6.6
HC 006	11.4	11.6	13.7	10.0	15.9	24.5	4.6	9.6
HC 001	14.9	9.0	12.8	9.2	15.5	23.3	6.0	6.0
HC 003	6.3	9.5	11.8	6.3	12.6	19.9	4.6	10.2
HC 004	9.3	7.6	10.3	5.8	12.2	20.1	4.5	7.2
HC 006	6.8	6.7	9.3	5.7	10.6	17.0	3.6	6.1
HC 001	11.2	9.5	11.6	7.6	14.7	23.2	6.1	10.0
HC 003	8.0	8.4	11.7	6.5	12.3	19.4	3.7	8.0
HC 001	19.4	11.9	15.8	8.9	15.1	26.6	7.5	15.3

Mean +/- SE for inside morphological measurements in millimeters for *Asarum contracta* (HC)

Location	Height	Distance	Hair length	Stigma lobes	Anther
HC 003	0.4	4.2	0.7	0.9	1.9
HC 004	0.5	4.8	1.0	0.9	2.3
HC 006	0.5	5.7	0.8	1.0	2.4
HC 001	0.4	7.1	0.9	0.6	2.5
HC 003	0.3	5.7	1.0	0.7	0.7
HC 004	0.3	4.0	0.8	0.4	0.4
HC 006	0.4	3.6	0.7	0.7	0.7
HC 001	0.3	6.7	1.1	0.7	1.8

Mean +/- SE for outside morphological measurements in millimeters for *Asarum rhombiformis* (HR)

Location	FA	FB	FC	FD	FE	FF	FLL	FLW
HR 001	8.7	7.7	12.1	9.4	14.4	19.8	4.1	6.5
HR 002	10.4	9.2	14.2	10.2	16.7	24.0	5.7	6.8
HR 003	13.8	11.1	15.5	11.8	19.2	25.6	5.6	5.0
HR 004	10.3	10.6	17.4	11.5	19.0	26.2	4.0	8.7
HR 006	9.4	9.7	15.9	10.3	16.8	23.0	5.2	.7.6
HR 007	13.6	12.2	17.8	10.9	10.6	27.2	6.2	11.4
HR 008	11.7	11.0	16.3	9.9	16.4	21.0	10.2	4.3
HR 010	11.4	9.6	15.0	12.6	20.5	26.9	6.2	8.3
HR 012	14.2	9.0	14.2	11.5	18.0	15.3	4.1	9.2
HR New	16.2	10.0	13.8	10.9	16.7	25.1	5.0	7.0
HR 001	10.8	12.0	16.0	17.0	15.2	20.6	4.4	9.0
HR 002	9.0	8.8	12.7	08.9	14.9	21.4	4.3	6.6
HR 003	11.8	10.6	14.3	10.6	16.1	24.9	4.8	9.5
HR 004	10.2	10.5	15.6	10.0	17.9	26.7	6.4	5.0
HR 006	8.7	8.6	15.6	08.6	14.5	20.9	4.8	5.7
HR 007	13.0	12.8	18.2	10.0	21.1	28.2	5.9	10.1
HR 008	12.3	12.6	18.5	12.0	19.7	27.7	6.7	9.1
HR 010	11.0	10.6	15.6	10.3	17.1	23.1	5.4	8.5
HR 012	9.2	9.6	14.2	8.2	14.8	19.9	5.0	7.1
HR New	10.9	10.2	14.9	10.3	15.4	24.5	6.4	8.2
HR 002	14.3	13.8	18.6	10.4	17.4	26.2	7.3	10.0
HR 004	7.6	7.6	12.9	8.8	14.5	18.8	2.4	2.8
HR 006	9.6	10.3	16.3	10.7	18.1	24.8	5.0	5.1
HR 007	11.0	9.1	14.2	11.1	19.0	24.5	5.4	8.1
HR 008	8.7	9.6	14.6	11.9	12.1	24.4	4.4	6.2
HR 010	9.3	8.5	13.2	9.2	13.5	18.0	3.9	6.5
HR 012	11.6	9.5	16.2	11.6	18.5	26.6	5.8	9.0
HR New	11.5	10.3	16.8	11.7	20.0	27.7	9.4	5.0

Mean +/- SE for inside morphological measurements in millimeters for *Asarum rhombiformis* (HR)

Location	Height	Distance	Hair	Stigma lobe	Anther
HR 001	1.1	6.8	0.2	3.0	1.9
HR 002	1.7	9.2	2.0	1.0	2.7
HR 004	1.4	8.6	4.0	2.3	2.9
HR 006	1.5	8.1	0.3	0.9	1.0
HR 007	1.5	9.0	4.0	1.1	2.3
HR 008	1.3	7.3	0.2	1.2	3.1
HR 010	1.0	8.4	0.3	1.5	2.8
HR 012	1.3	7.9	0.2	1.9	3.1
HR New	1.0	8.3	0.3	1.3	2.4

Morphological measurements in centimeters corrected from Carroll (1996).

ID	FA	FB	FC	FD	FE	FF
hc1	0.50	1.30	1.60	1.00	1.10	2.90
hc2	0.50	1.00	1.30	0.90	1.20	2.10
h3	0.90	1.00	1.50	1.10	1.30	2.00
hc4	1.10	1.10	1.80	0.90	1.40	2.80
hc5	0.70	1.10	1.20	1.00	1.40	3.10
hc6	0.40	1.20	1.10	0.70	1.10	2.30
hc7	1.00	1.00	1.30	0.80	1.00	1.90
hc8	1.10	1.30	1.30	0.90	1.30	2.60
hc9	0.90	0.90	1.10	0.90	1.30	1.50
hc10	0.30	0.90	0.80	0.60	0.90	1.50
hc11	1.20	1.40	1.50	0.90	1.10	2.90
hc12	1.10	1.60	1.30	0.80	1.00	3.10
hc13	1.30	1.00	1.10	0.70	1.10	2.10
hc14	0.50	2.60	0.80	0.70	1.10	2.00
hc15	0.80	1.20	0.70	0.80	0.90	2.50

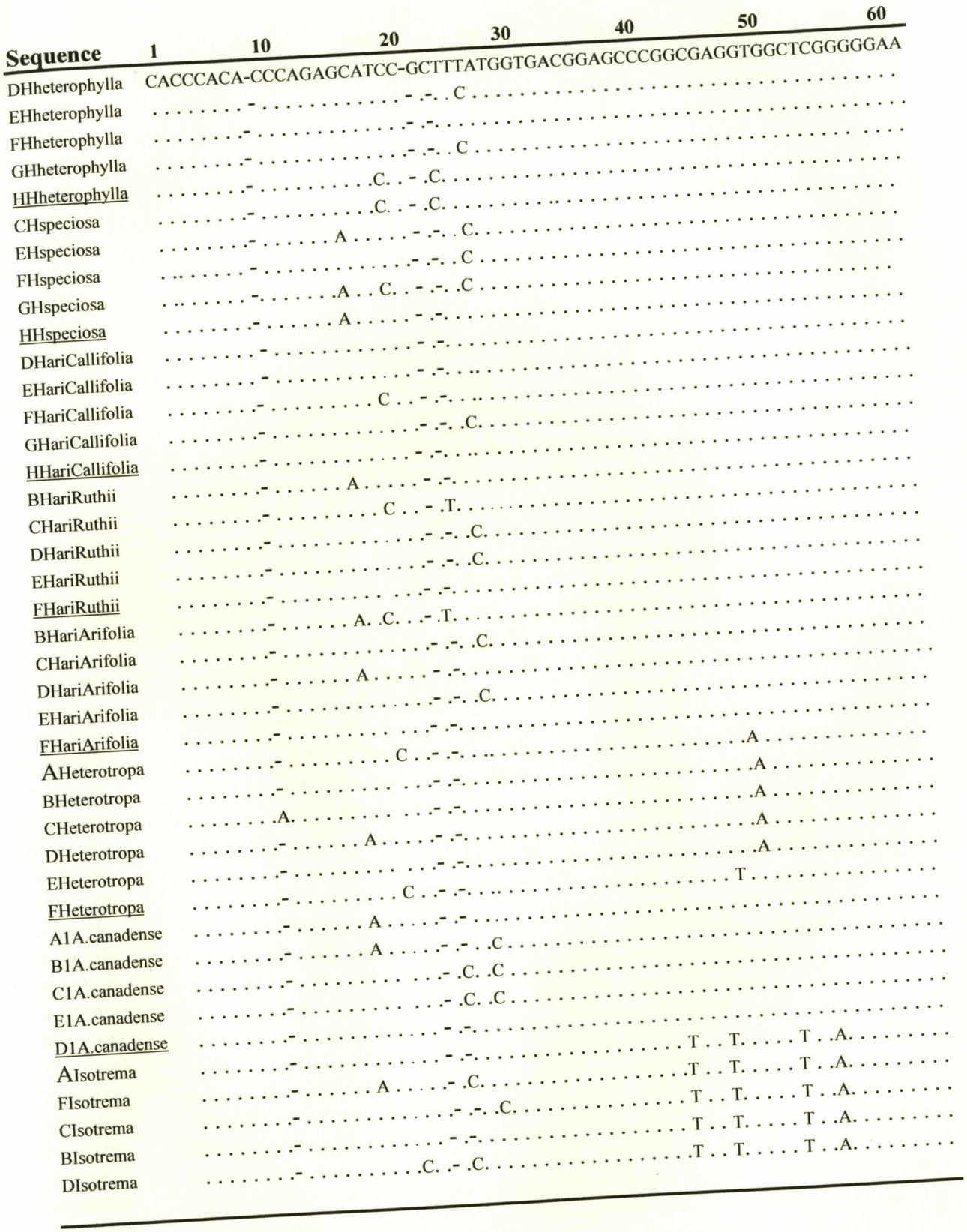
APPENDIX D

Molecular Sequences

Sequence	1	10	20	30	40	50	60
B1HC 001	CACCCACA-CCCAGAGCATCC-G-TTCATGGTGACGGAGCCCGGTGAGGTGGCTCGGGGGAA						
C1HC 001	. . . . . .-. . . . . C . .- .T.						
E1HC 001	. . . . . .-. . . . .- .-.						
D1HC 001	. . . . . .-. . . . .- .-.						
F1HC 001	. . . . . .-. . . . .- .-.						
A2HC 001	. . . . . .-. . . . .- .-. . .T.						
D2HC 001	. . . . . .-. . . . . C . .- .-. . .T.						
E2HC 001	. . . . . .-. . . . .- .-. . .T.						
F2HC 001	. . . . . .-. . . . .- .-.						
G2HC 001	. . . . . .-. . . . .- .-.						
A1HC 003	. . . . . .-. . . . . A . . . . .- .C. . . . . .C						
B1HC 003	. . . . . .-. . . . .- .-. . .T.						
C1HC 003	. . . . . .-. . . . .- .-. . .T. . . . . .C.						
F1HC 003	. . . . . .-. . . . .- .C . .T.						
G1HC 003	. . . . . .-. . . . . C . .- .C . .T.						
A2HC 003	. . . . . .-. . . . . A . . . . .- -C. . .T.						
B2HC 003	. . . . . .-. . . . .- .C. . . . . .C.						
C2HC 003	. . . . . .-. . . . .- .-.						
D2HC 003	. . . . . .-. . . . .- .-. . . . . .C.						
E2HC 003	. . . . . .-. . . . . C . .- .C . . . . . .C.						
B2HC 004	. . . . . .-. . . . .- .-.						
C2HC 004	. . . . . .-. . . . . A . . . . -. - . . . . . .C						
A2HC 004	. . . . . .-. . . . . C . .- .- . .T. . . . . .C						
D2HC 004	. . . . . .-. . . . .- .-. . .T.						
A2HC 004	. . . . . .-. . . . . C . .- .- . .T. . . . . .C						
E2HC 004	. . . . . .-. . . . . A . . . . .- .-. . . . . .C						
B3HC 004	. . . . . .-. . . . .- .-. . .T. . . . . .C						
C3HC 004	. . . . . .-. . . . .- .C . .T. . . . . .C						
D3HC 004	. . . . . .-. . . . . C . .- .C . . . . . .C						
E3HC 004	. . . . . .-. . . . .- .C . .T.						
F3HC 004	. . . . . .-. . . . .- .C . .T. . . . . .C.						
A1HC 006	. . . . . .-. . . . .- .-. . .T.						
B1HC 006	. . . . . .-. . . . . C . .- .-. . .T.						

Sequence	1	10	20	30	40	50	60
B1HCR006	CACCCACA-CCCAGAGCACCC-G-TTTATGGTGACGGAGCCCGGTGAGGTGGCTCGGGGGAA						
B1HC 006	. G . . . . . .- . . . . . T . . - .-.						
C1HC 006	. . . . . .- . . . . . .T . .- .C. .C. . . . . C.						
D1HC 006	. . . . . .- . . . . . A. . .T . .- .-						
H1HC 006	. . . . . .- . . . . . A. . .T. . - .-. . . . . C.						
F1HC 006	. . . . . .- . . . . . - .-.						
E1HC 006	. . . . . - . . . . . T . . - .-.						
--2HC 006	. . . . . - . . . . . T . . - .-. .C . . . . . C						
E2HC 006	. . . . . .- . . . .- .C.						
--2HC 006	. . . . . .- . . . .- .-.						
D2HCr006	. . . . . .- . . . . . - .-. .C						
C2HC 006	. . . . . .- . . . .- .-.						
A1HC TN	. . . . . .- . . . . . T . .- .-.						
C1HC TN	. . . . . .- . . . . . .- . .-. .C						
D1HC TN	. . . . . .- . . . . . .- .-.						
E1HC TN	. . . . . .- . . . . . A . .T. .- .CC.C.						
F1HC TN	. . . . . .- . . . . . T. .-.CCC. T-						
C2HC TN	. . . . . .- . . . . . A. .-.C. . . . . C						
D2HC TN	. . . . . .- . . . . . T . .- .-. . . . . .C						
F2HC TN	. . . . . .- . . . . . T . .- .-. . . . . .C						
G2HC TN	. . . . . .- . . . . . T . .C.-. . . . . .C						
H2HC TN	. . . . . .- . . . . . T . .- .-. . . . . .C						
C1HR 004	. . . . . .- . . . . . T . .- .-.						
G1HR 004	. . . . . .- . . . . . T . .- .C.						
A1HR 004	. . . . . .- . . . . . .- . C.						
E1HR 004	. . . . . .- . . . . . T . .- .C.						
D1HR 004	. . . . . .- . . . . . T . .- .-.						
G2HR 004	. . . . . .- . . . . . T . .- .-. . C . . . . . .C.						
D2HR 004	. . . . . .- . . . . . T . .- .-						
E2HR 004	. . . . . .- . . . . . T . .- .-. . C . . . . . .C.						
H2HR 004	. . . . . .- . . . . . A . . T . .- .T. . . . . .C.						
C2HR 004	. . . . . .- . . . . . T . .- .-. . . . . .C.						
F2HR 004	. . . . . .- . . . . . A . . . . .- .- . . . . . C						
C1HR 007	. . . . . .- . . . . . .- . C.						
A1HR 007	. . . . . .- . . . . . A . . T . .- .-.						
B1HR 007	. . . . . .- . . . . . A . . . . .- .C						
D1HR 007	. . . . . .- . . . . . T . .- .C.						
E1HR 007	. . . . . .- . . . . . A. . T . .- .-.						
A2HR 007	. . . . . .- . . . . . .- . C.						
B2HR 007	. . . . . .- . . . . . A . . T . .- .-. .C.						
C2HR 007	. . . . . .- . . . . . .- .-.						
E2HR 007	. . . . . .- . . . . . A. . T . .- .-.						
G2HR 007	. . . . . .- . . . . . .- .-.						
H2HR 007	. . . . . .- . . . . . T . .- .-. . C						
B1HR 010	. . . . . .- . . . . . T . .C .C. . . . . .C.						

Sequence	1	10	20	30	40	50	60
F1HR 010	CACCCACA-CCCAGAGCATCC-G-TTCATGGTGACGGAGCCCGGCGAGGTGGCTCGGGGGAA						
A1HR 010	. . . . . .-. . . . . A. . -. . C.C . .T. . . . . T . . . . .
1HR 0101	. . . . . .-. . . . . C . .- .- . .T. . . . .
1HR 0100	. . . . . .-. . . . . C . .- .- . . T. . . . .
--2HR 0010	. . . . . .-. . . . . - .- . .T. . . . .
D2HR 010	. . . . . .-. . . . . C . .- .-. . T. . . . . T . . . . .
B2HR 010	. . . . . .-. . . . . .. - .-. . T. . . . .
--2HR 010	. . . . . .-. . . . . - .-. .T . . . . .
C2HRR010	. . . . . .-. . . . . . .- .-. . T . . . . .
C2HR 010	. . . . . .-. . . . . . .- .-. . . . .
G1HR 012	. . . . . .-. . . . . A . . . . . - .-. .T . . . . .
E1HR 012	. . . . . .-. . . . . . . -.CC .T . . . . .
B1HR 012	. . . . . .-. . . . . C . . - .-. . .. .
C1HR 012	. . . . . .-. . . . . .- .-. .T. . . . .
D1HR 012	. . . . . .-. . . . . .- .T. . . . . G
--2HR 012	. . . . . .-. . . . . A . . . . . - .-. .T. . . . . T . . . . .
C2HR 012	. . . . . .-. . . . . C . . - .-. .T. . . . . T . . . . .
E2HR 012	. . . . . .-. . . . . C . . -. -. .T. . . . . T . . . . .
D2HR 012	. . . . . .-. . . . . .- .-. .T. . . . .
B2HR 012	. . . . . .-. . . . . . .- .-. . . . .
C1Hvirginica	. . . . . .-. . . . . . .- .-. . . . . T . . . . .
B1Hvirginica	. . . . . .-. . . . . A . . . . .- .G. .T. . . . . T . . . . .
A1Hvirginica	. . . . . .-. . . . . A. . C. . .- .-. . . . . T. . . . .
D1Hvirginica	. . . . . .-. . . . . C . . -. -. .T. . . . . T . . . . .
E1Hvirginica	. . . . . .-. . . . . . .- .-. . . . . T. . . . .
AHnaniflora	. . . . . .-. . . . . C . . -. -. .T. . . . . T . . . . .
DHnaniflora	. . . . . .-. . . . . C . . -. -. .T. . . . . T . . . . .
CHnaniflora	. . . . . .-. . . . . C . . -. -. .T. . . . .
GHnaniflora	. . . . . .-. . . . . C . . - .T. . .CG. . . . .
EHnaniflora	. . . . . .-. . . . . A . . . . . - .C. . . . . T. . . . .
C1Hlewisii	. . . . . .-. . . . . .. -. -. .T. . . . . T. . . . .
BHlewisii	. . . . . .-. . . . . A . . . . .- .- . . . . . T. . . . .
AHlewisii	. . . . . .-. . . . . A . . . . .- .- . .T . . . . . T . . . . .
EHlewisii	. . . . . .-. . . . . . .- .-. . . . . T . . . . .
DHlewisii	. . . . . .-. . . . . . .- .-. . T . . . . . T . . . . .
EHshuttlew	. . . . . .-. . . . . C . . - .-. . .. .
HHshuttlew	. . . . . .-. . . . . A . . . . .- .- . .T . . . . .
GHshuttlew	. . . . . .-. . . . . . .- .-. . T . . . . .
DHshuttlew	. . . . . .-. . . . . A. . C . . -. -. .T. . . . .
CHshuttlew	. . . . . .-. . . . . . .- .-. . T . . . . .
AHminor	. . . . . .-. . . . . . .C.-. . T . . . . .
BHminor	. . . . . .-. . . . . A . . . . .- .-. . . . .
CHminor	. . . . . .-. . . . . .. -. -. .T. . . . .
DHminor	. . . . . .-. . . . . .. -. -. .T. . . . .
EHminor	. . . . . .-. . . . . A . . . . .- .- . .T . . . . . T . . . . .


Sequence
1
10
20
30
40
50
60
DHheterophylla CACCCACA-CCCAGAGCATCC-GCTTTATGGTGACGGAGCCCGGCGAGGTGGCTCGGGGGAA
EHheterophylla
FHheterophylla
GHheterophylla
HHheterophylla
CHspeciosa
EHspeciosa
FHspeciosa
GHspeciosa
HHspeciosa
DHariCallifolia
EHariCallifolia
FHariCallifolia
GHariCallifolia
HHariCallifolia
BHariRuthii
CHariRuthii
DHariRuthii
EHariRuthii
FHariRuthii
BHariArifolia
CHariArifolia
DHariArifolia
EHariArifolia
FHariArifolia
AHeterotropa
BHeterotropa
CHeterotropa
DHeterotropa
EHeterotropa
FHeterotropa
A1A.canadense
B1A.canadense
C1A.canadense
E1A.canadense
D1A.canadense
AIsotrema
FIsotrema
CIsotrema
BIsotrema
DIsotrema

Sequence	63	70	80	90	100	110	120 125
B1HC 001	GAAGAATGGACTGGATTATC-TCTTCCACCTCTACGAGCAATGCCGGGAGTTCTGCTTCAGG						
C1HC 001	.						
E1HC 001	.						
D1HC 001	.						
F1HC 001	.						
A2HC 001	.						
D2HC 001	.						
E2HC 001	.						
F2HC 001	.						
G2HC 001	.						
A1HC 003	.						
B1HC 003	.			.C.			
C1HC 003	.						
F1HC 003	.						
G1HC 003	.						
A2HC 003	.						
B2HC 003	.						
C2HC 003	.						
D2HC 003	.						
E2HC 003	.						
B2HC 004	.						
C2HC 004	.						
A2HC 004	.						
D2HC 004	.						
A2HC 004	.						
E2HC 004	.						
B3HC 004	.			.G.			
C3HC 004	.						
D3HC 004	.						
E3HC 004	.						
F3HC 004	.						
A1HC 006	.						
B1HC 006	.						
B1HC 006	.						
B1HCR006	.						
B1HC 006	.						
C1HC 006	.						
D1HC 006	.						
H1HC 006	.						
F1HC 006	.						
E1HC 006	.						
--2HC 006	.						
E2HC 006	.						
--2HC 006	.						
D2HCr006	.			.C			

Sequence	63	70	80	90	100	110	120 125
C2HC 006	GAAGAATGGACTGGATTATC-TCTTCCACCTCTACGAGCAATGCCGGGAGTTCCTGCTTCAGG						
A1HC TN	. . . . .						
C1HC TN	. . . . .						
D1HC TN	. . . . .						
E1HC TN	. . . . .						
F1HC TN	. . . . .		G.	. . . . .			G. .
C2HC TN	. . . . .						
D2HC TN	. . . . .						
F2HC TN	. . . . .						
G2HC TN	. . . . .						
H2HC TN	. . . . .						
C1HR 004	. . . . .						
G1HR 004	. . . . .						
A1HR 004	. . . . .						
E1HR 004	. . . . .						
D1HR 004	. . . . .						
G2HR 004	. . . . .						
D2HR 004	. . . . .						
E2HR 004	. . G.	. . . . .					
H2HR 004	. . . . .						
C2HR 004	. . . . .	A.	. . . . .				
F2HR 004	. . . . .						
C1HR 007	. . . . .						
A1HR 007	. . . . .				A.	. . . . .	
B1HR 007	. . . . .						
D1HR 007	. . . . .						
E1HR 007	. . . . .						
A2HR 007	. . . . .						
B2HR 007	. . . . .						
C2HR 007	. . . . .						
E2HR 007	. . . . .						
G2HR 007	. . . . .						
H2HR 007	. . . . .						
B1HR 010	. . . . .				G.	. . . . .	
F1HR 010	. . . . .						
A1HR 010	. . . . .						
1HR 0101	. . . . .						
1HR 0100	. . . . .						
--2HR 0010	. . . . .						
D2HR 010	. . . . .						
B2HR 010	. . . . .						
--2HR 010	. . . . .						
C2HR 010R	. . . . .						
C2HR 010	. . . . .				G.	. . . . .	
G1HR 012	. . . . .						

Sequence	63	70	80	90	100	110	120 125
E1HR 012	GAAGAATGGACTGGATTATC-TCTTCCACCTCTACGAGCAATGCCGGGAGTTCCTGCTTCAGG						
B1HR 012	.						
C1HR 012	.						
D1HR 012	.						
--2HR 012	.						
C2HR 012	.						
E2HR 012	.						
D2HR 012	.						
B2HR 012	.						
C1Hvirginica	.						
B1Hvirginica	.						
A1Hvirginica	.						
D1Hvirginica	.						
E1Hvirginica	.						
AHnaniflora	A.						
DHnaniflora	A.						
CHnaniflora	.						
GHnaniflora	.						
EHnaniflora	.						
C1Hlewisii	.						
B1Hlewisii	.						
A1Hlewisii	.						
E1Hlewisii	.						
D1Hlewisii	. . . . G.						
EHshuttlew	.						
HHshutttlew	.						
GHshutttlew	.						
DHshutttlew	.						
CHshutttlew	.						
AHminor	.						
BHminor	.						
CHminor	.						
DHminor	.						
EHminor	.				.G		
DHheterophylla	.						
EHheterophylla	.						
FHheterophylla	.						
GHheterophylla	.						
HHheterophylla	.						
CHspeciosa	.						
EHspeciosa	. . . . .G.						
FHspeciosa	.						
GHspeciosa	.						
HHspeciosa	.						
DHariCallifolia	.						

Sequence	63	70	80	90	100	110	120 125
EHariCallifolia	GAAGAATGGACTGGATTATC-TCTTCCACCTCTACGAGCAATGCCGGGAGTTCCTGCTTCAGG						
FHariCallifolia	. . . . .						
GHariCallifolia	. . . . .						
HHariCallifolia	. . . . .						
BHariRuthii	. . . . .						
CHariRuthii	. . . . .						
DHariRuthii	. . . . .						
EHariRuthii	. . . . .						
FHariRuthii	. . . . .						
BHariArifolia	. . . . .						
CHariArifolia	. . . . .		.C	. . . . .			
DHariArifolia	. . . . .						
EHariArifolia	. . . . .						
FHariArifolia	. . . . .						
AHeterotropa	. . . . .					T.	. . . . .
BHeterotropa	. . . . .					T.	. . . . .
CHeterotropa	. . . . .					T.	. . . . .
DHeterotropa	. . . . .					T.	. . . . .
EHeterotropa	. . . . .					T.	. . . . .
FHeterotropa	. . . . .					T.	. . . . .
A1A.canadense	. . . . .						
B1A.canadense	. . . . .						
C1A.canadense	. . . . .						
E1A.canadense	. . . . .						
D1A.canadense	. . . . .						
AIsotrema	. . .A. . . . .GT.	. . . . .			G.	G.	C. . C. . . .
FIsotrema	. . .A. . . . .GT.	. . . . .			G.	G.	C. . C. . . .
CIsotrema	. . .A. . . . .GT.	. . . . .			G.	G.	C. . C. . . .
BIsotrema	. . .A. . . . .GT.	. . . . .			G.	G.	C. . C. . . .
DIsotrema	. . .A. . . . .GT.	. . . . .			G.	G.	C. . C. . . .

Sequence	126	130	140	150	160	170	180	185
B1HC 001	TCCAGGCTATTGCCAAAGAGAAGGGCGAGAAGTGCCCCACCAAGGTAAATAGTAATCGGA							
C1HC 001	.							
E1HC 001	.							
D1HC 001	.							
F1HC 001	.							
A2HC 001	.							
D2HC 001	.							
E2HC 001	.							
F2HC 001	.							
G2HC 001	.							
A1HC 003	.					-		
B1HC 003	.							
C1HC 003	.					-		
F1HC 003	.							
G1HC 003	.							
A2HC 003	.							
B2HC 003	.					-		
C2HC 003	.					-		
D2HC 003	.					-		
E2HC 003	.					-		A.
B2HC 004	.					-		
C2HC 004	.					-		
A2HC 004	.					-		
D2HC 004	.					-		
A2HC 004	.					-		
E2HC 004	.					-		
B3HC 004	.					-		
C3HC 004	.					-		
D3HC 004	.					-		
E3HC 004	.							
F3HC 004	.					-		
A1HC 006	.					-		
B1HC 006	.							A.
B1HC 006	.					-		
A1HCR006	.							A.
B1HC 006	.							
C1HC 006	.							
D1HC 006	.							
H1HC 006	.							
F1HC 006	.							
E1HC 006	.					-		
--2HC 006	.							
E2HC 006	.					-		
--2HC 006	.					-		
D2HCr006	.							

Sequence	126	130	140	150	160	170	180	185
C2HC 006	TCCAGGCTATTGCCAAAGAGAAGGGCGAGAAGTGCCCCACCAAGGTAAATAGTAATCGGA							
A1HC TN	.							
C1HC TN	.							
D1HC TN	.							
E1HC TN	.							
F1HC TN	.							
C2HC TN	.					-		
D2HC TN	.					-		
F2HC TN	.					-		
G2HC TN	.					-		
H2HC TN	.					-		
C1HR 004	.							
G1HR 004	.							
A1HR 004	.							
E1HR 004	.							
D1HR 004	.							
G2HR 004	.					-		
D2HR 004	.							
E2HR 004	.					-		
H2HR 004	.					-		
C2HR 004	.							
F2HR 004	.					-		
C1HR 007	.					C		
A1HR 007	.							
B1HR 007	.							
D1HR 007	.							
E1HR 007	.							
A2HR 007	.							
B2HR 007	.							
C2HR 007	.							
E2HR 007	.							
G2HR 007	.							
H2HR 007	.							
B1HR 010	.							
F1HR 010	.							
A1HR 010	.							
1HR 0101	.							
1HR 0100	.							
--2HR 0010	.					-		
D2HR 010	.			G				
B2HR 010	.					-		
--2HR 010	.					-		
C2HR 010R	.					-		
C2HR 010	.					-		
G1HR 012	.					-		

Sequence	126	130	140	150	160	170	180	185
B1HR 012	TCCAGGCTATTGCCAAAGAGAAGGGCGAGAAGTGCCCCACCAAGGTAA-TAGTAATCGGA							
E1HR 012	. . . . .							.A. .
C1HR 012	. . . . .							. . . .
D1HR 012	. . . . .							. . . .
--2HR 012	. . . . .					.A	. . . . .	
C2HR 012	. . . . .					.A	. . . . .	
E2HR 012	. . . . .					.A	. . . . .	..
D2HR 012	. . . . .					.A	. . . . .	..
B2HR 012	. . . . .							. . . .
C1Hvirginica	. . . . .					.A	. . . . .	
B1Hvirginica	. . . . .					.A	. . . . .	
A1Hvirginica	. . . . .					.A	. . . . .	..
D1Hvirginica	. . . . .					.A	. . . . .	
E1Hvirginica	. . . . .					.A	. . . . .	..
AHnaniflora	. . . . .					.A	. . . . .	
DHnaniflora	. . . . .					.A	. . . . .	
CHnaniflora	. . . . .							. . . .
GHnaniflora	. . . . .					.A	. . . . .	
EHnaniflora	. . . . .					.A	. . . . .	
C1Hlewisii	. . . . .							. . . .
B1Hlewisii	. . . . .							. . . .
A1Hlewisii	. . . . .							. . . .
E1Hlewisii	. . . . .							. . . .
D1Hlewisii	. . . . .							. . . .
EHshuttlew	. . . . .							. . . .
HHshutttlew	. . . . .							. . . .
GHshutttlew	. . . . .							. . . .
DHshutttlew	. . . . .							. . . .
CHshutttlew	. . . . .							. . . .
AHminor	. . . . .							. . . .
BHminor	. . . . .							. . . .
CHminor	. . . . .							. . . .
DHminor	. . . . .							. . . .
EHminor	. . . . .							. . . .
DHheterophylla	. . . . .							. . . .
EHheterophylla	. . . . .							. . . .
FHheterophylla	. . . . .		G.	. . . . .				. . . .
GHheterophylla	. . . . .							. . . .
HHheterophylla	. . . . .							. . . .
CHspeciosa	. . . . .							. . . .
EHspeciosa	. . . . .							. . . .
FHspeciosa	. . . . .							. . . .
GHspeciosa	. . . . .							. . . .
HHspeciosa	. . . . .							. . . .
DHariCallifolia	. . . . .							. . . .

Sequence	126	130	140	150	160	170	180	185
EHariCallifolia	TCCAGGCTATTGCCAAAGAGAAGGGCGAGAAGTGCCCCACCAAGGTAA-TAGTAATCGGA							
FHariCallifolia	. . . . .							
GHariCallifolia	. . . . .						- . A . . - - .	
HHariCallifolia	. . . . .							
BHariRuthii	. . . . .							
CHariRuthii	. . . . .							
DHariRuthii	. . . . .							
EHariRuthii	. . . . .							A .
FHariRuthii	. . . . .							A .
BHariArifolia	. . . . .							
CHariArifolia	. . . . .							
DHariArifolia	. . . . .							
EHariArifolia	. . . . .							
FHariArifolia	. . . . .							
AHeterotropa	. T				A		G	
BHeterotropa	. T				A		G	
CHeterotropa	. T				A		G	
DHeterotropa	. T				A		G	
EHeterotropa	. T				A		G	
FHeterotropa	. . . . .					T		
A1A.canadense	. . . . .	. T			A		G	
B1A.canadense	. . . . .	. T			A		G	
C1A.canadense	. . . . .	. T			A		G	
E1A.canadense	. . . . .	. T			A		G	
D1A.canadense	. . . . .	. T			A		G	
AIsotrema	C . . . AAC . GC . . T . . G						- . A . . - - .	
FIsotrema	C . . . AAC . GC . . T . . G						- . A . . - - .	
CIsotrema	C . . . AAC . GC . . T . . G						- . A . . - - .	
BIsotrema	C . . . AAC . GC . . T . . G						- . A . . - - .	
DIsotrema	C . . . AAC . GC . . T . . G						- . A . . - - .	

Sequence	186 190	200	210	220	230	240	246
B1HC 001	TCCTTACATTAAGCGGGCTTGTTC	- - - -	GGGTAATTAAATTTTAGATCCAATT	-	ATTTAGA		
C1HC 001	. . . . .						
E1HC 001	. . . . .						
D1HC 001	. . . . .						
F1HC 001	. . . . .						
A2HC 001	. . . . .						
D2HC 001	. . . . .						
E2HC 001	. . . . .						
F2HC 001	. . . . .						
G2HC 001	. . . . .						
A1HC 003	. . . . .						
B1HC 003	. . . . .						
C1HC 003	. . . . .						
F1HC 003	. . . . .		C.	. . . . .			
G1HC 003	. . . . .						
A2HC 003	. . . . .						
B2HC 003	. . . . .						
C2HC 003	. . . . .						
D2HC 003	. . . . .						
E2HC 003	. . . . .						
B2HC 004	. . . . .						
C2HC 004	. . . . .						
A2HC 004	. . . . .						
D2HC 004	. . . . .						
A2HC 004	. . . . .						
E2HC 004	. . . . .						
B3HC 004	. . . . .						
C3HC 004	. . . . .						
D3HC 004	. . . . .						
E3HC 004	. . . . .						
F3HC 004	. . . . .						
A1HC 006	. . . . .						
B1HC 006	. . . . .						
B1HC 006	. . . . .						
B1HCR006	. . . . .						
B1HC 006	. . . . .						
C1HC 006	. . . . .						
D1HC 006	. . . . .						
H1HC 006	. . . . .						
F1HC 006	. . . . .						
E1HC 006	. . . . .						
--2HC 006	. . . . .						
E2HC 006	. . . . .						
--2HC 006	. . . . .						
D2HCr006	. . . . .						

Sequence	186 190	200	210	220	230	240	245
C2HC 006	TCCTTACATTAAGCGGGCTTGTTC	- - - -	GGGTAATTAAATTTTAGATCCAATT	-	ATTTAGA		
A1HC TN	. . . . .						
C1HC TN	. . . . .						
D1HC TN	. . . . .						
E1HC TN	. . . . .						
F1HC TN	. . . . .						
C2HC TN	. . . . .						
D2HC TN	. . . . .						
F2HC TN	. . . . .						
G2HC TN	. . . . .						
H2HC TN	. . . . .						
C1HR 004	. . . . .						
G1HR 004	. . . . .						
A1HR 004	. . . . .						
E1HR 004	. . . . .						
D1HR 004	. . . . .						
G2HR 004	. . . . .						
D2HR 004	. . . . .						
E2HR 004	. . . . .						
H2HR 004	. . . . .						
C2HR 004	. . . . .						
F2HR 004	. . . . .						
C1HR 007	. . . . .						
A1HR 007	. . . . .						
B1HR 007	. . . . .						
D1HR 007	. . . . .						
E1HR 007	. . . . .						
A2HR 007	. . . . .						
B2HR 007	. . . . .						
C2HR 007	. . . . .						
E2HR 007	. . . . .						
G2HR 007	. . . . .						
H2HR 007	. . . . .						
B1HR 010	. . . . .						
F1HR 010	. . . . .						
A1HR 010	. . . . .						
1HR 0101	. . . . .						
1HR 0100	. . . . .						
--2HR 0010	. . . . .						
D2HR 010	. . . . .					C.	. . . . .
B2HR 010	. . . . .	.C.	. . . . .				
--2HR 010	. . . . .						
C2HR 010R	. . . . .	.C.	. . . . .				
C2HR 010	. . . . .						
G1HR 012	. . . . .						

Sequence	186 190	200	210	220	230	240	245
E1HR 012	TCCTTACATTAAGCGGGCTTGTTC	- - - -	GGGTAATTAAATTTTAGATCCAATT	-	ATTTAGA		
B1HR 012	. . . . .						
C1HR 012	. . . . .						
D1HR 012	. . . . .						
--2HR 012	. . . . .						
C2HR 012	. . . . .						
E2HR 012	. . . . .						
D2HR 012	. . . . .						
B2HR 012	. . . . .						
C1Hvirginica	. . . . .						
B1Hvirginica	. . . . .						
A1Hvirginica	. . . . .						
D1Hvirginica	. . . . .						
E1Hvirginica	. . . . .						C. . . .
AHnaniflora	. . . . .						
DHnaniflora	. . . . .						
CHnaniflora	. . . . .						
GHnaniflora	. . . . .						
EHnaniflora	. . . . .						
C1Hlewisii	. . . . .						
B1Hlewisii	. . . . .						
A1Hlewisii	. . . . .						
E1Hlewisii	. . . . .						
D1Hlewisii	. . . . .						
EHshuttlew	. . . . .						
HHshutttlew	. . . . .						
GHshutttlew	. . . . .						
DHshutttlew	. . . . .						
CHshutttlew	. . . . .						
AHminor	. . . . .						
BHminor	. . . . .						
CHminor	. . . . .						
DHminor	. . . . .						
EHminor	. . . . .			.C.	. . . . .		
DHheterophylla	. . . . .						
EHheterophylla	. . . . .						
FHheterophylla	. . . . .						
GHheterophylla	. . . . .						
HHheterophylla	. . . . .						
CHspeciosa	. . . . .						
EHspeciosa	. . . . .						
FHspeciosa	. . . . .						
GHspeciosa	. . . . .						
HHspeciosa	. . . . .						
DHariCallifolia	. . . . .						

Sequence	186 190	200	210	220	230	240	245
EHariCallifolia	TCCTTACATTAAGCGGGCTTGTTC	- - - -	GGGTAATTAAATTTTAGATCCAATT	-	ATTTAGA		
FHariCallifolia	. . . . .	. . . . .	. . . . .	. . . . .	.C.	. . . . .	. . . . .
GHariCallifolia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
HHariCallifolia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
BHariRuthii	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
CHariRuthii	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
DHariRuthii	. . . . .	.G.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
EHariRuthii	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
FHariRuthii	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
BHariArifolia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
CHariArifolia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
DHariArifolia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
EHariArifolia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
FHariArifolia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.C	. . . . .
AHeterotropa	. . . . .	. . . . .	A.	. . . . .	. . . . .	.C	. . . . .
BHeterotropa	. . . . .	. . . . .	A.	. . . . .	. . . . .	.C	. . . . .
CHeterotropa	. . . . .	. . . . .	A.	. . . . .	. . . . .	.C	. . . . .
DHeterotropa	. . . . .	. . . . .	A.	. . . . .	. . . . .	.C	. . . . .
EHeterotropa	. . . . .	. . . . .	A.	. . . . .	. . . . .	.C. . . .	. . . . .
FHeterotropa	. . . . .	. . . . .	A.	. . . . .	. . . . .	. . . . .	. . . . .
A1A.canadense	. . . . .	. . . . .	. . . . .	A. . . .	A.	. . . . .	. . . . .
B1A.canadense	. . . . .	. . . . .	C.	A. . . .	A.	. . . . .	. . . . .
C1A.canadense	. . . . .	. . . . .	. . . . .	A. . . .	A.	. . . . .	. . . . .
E1A.canadense	. . . . .	. . . . .	. . . . .	A. . . .	A.	. . . . .	. . . . .
D1A.canadense	. . . . .	. . . . .	. . . . .	A. . . .	A.	. . . . .	. . . . .
AIsotrema	CGCAATCAATCTTCTT	-	.TTCA .A . .	CT. C. TTC	- - - - - - -	. . . . .	.C. . . GGT . .
FIsotrema	CGCAATCAATCTTCTT	-	.TTCA .A . .	CT. C. TTC	- - - - - - -	. . . . .	.C. . . GGT . .
CIsotrema	CGCAATCAATCTTCTT	-	.TTCA .A . .	CT. C. TTC	- - - - - - -	. . . . .	.C. . . GGT . .
BIsotrema	CGCAATCAATCTTCTT	-	.TTCA .A . .	CT. C. TTC	- - - - - - -	. . . . .	.C. . . GGT . .
DIsotrema	CGCAATCAATCTTCTT	-	.TTCA .A . .	CT. C. TTC	- - - - - - -	. . . . .	.C. . . GGT . .

Sequence	311 315	320	330	340	350	360	370
B1HC 001	AACCAGGTGTTTCGATATGCAAAGAAGGCAGGGGCGAGCTACATTAACAAACC - AAAAT						
C1HC 001	. . . . .						
E1HC 001	. . . . .					G	
D1HC 001	. . . . .						
F1HC 001	. . . . .						
A2HC 001	. . . . .					G	G. .
D2HC 001	. . . . .						
E2HC 001	. . . . .						
F2HC 001	. . . . .					G	
G2HC 001	. . . . .					G	
A1HC 003	. . . . .					G	
B1HC 003	. . . . .						
C1HC 003	. . . . .					G	
F1HC 003	. . . . .					G	G. .
G1HC 003	. . . . .					G	G. .
A2HC 003	. . . . .					G	G. .
B2HC 003	. . . . .						
C2HC 003	. . . . .						G. .
D2HC 003	. . . . .						G. .
E2HC 003	. . . . .						G. .
B2HC 004	. . . . .						G. .
C2HC 004	. . . . .					G	
A2HC 004	. . . . .						
D2HC 004	. . . . .						
A2HC 004	. . . . .						
E2HC 004	. . . . .					G	
B3HC 004	. . . . .						G. .
C3HC 004	. . . . .					G	
D3HC 004	. . . . .						G. .
E3HC 004	. . . . .						
F3HC 004	. . . . .						G. .
A1HC 006	. . . . .						
B1HC 006	. . . . .						G. .
B1HC 006	. . . . .						
B1HCR006	. . . . .						G. .
B1HC 006	. . . . .						
C1HC 006	. . . . .					G	
D1HC 006	. . . . .						
H1HC 006	. . . . .						
F1HC 006	. . . . .					G	G. .
E1HC 006	. . . . .						
--2HC 006	. . . . .				T	G	G. .
E2HC 006	. . . . .					G.	
--2HC 006	. . . . .					G	G. .
D2HCr006	. . . . .						G. .

Sequence	311 315	320	330	340	350	360	370
C2HC 006	AACCAGGTGTTTCGATATGCAAAGAAGGCAGGGGCGAGCTACATTAACAAACCC - AAAAT						
A1HC TN	. . . . .						
C1HC TN	. . . . .					G	
D1HC TN	. . . . .						
E1HC TN	. . . . .						
F1HC TN	. . . . .						G. .
C2HC TN	. . . . .						
D2HC TN	. . . . .					.G	
F2HC TN	. . . . .						G. .
G2HC TN	. . . . .						G. .
H2HC TN	. . . . .	A.					
C1HR 004	. . . . .						
G1HR 004	. . . . .						
A1HR 004	. . . . .						
E1HR 004	. . . . .					.G	
D1HR 004	. . . . .					.G	
G2HR 004	. . . . .						
D2HR 004	. . . . .						
E2HR 004	. . . . .						
H2HR 004	. . . . .						
C2HR 004	. . . . .						
F2HR 004	. . . . .						G. .
C1HR 007	. . . . .						G. .
A1HR 007	. . . . .					.G	G. .
B1HR 007	. . . . .						
D1HR 007	. . . . .						
E1HR 007	. . . . .						
A2HR 007	. . . . .					.G	
B2HR 007	. . . . .					.G	
C2HR 007	. . . . .					.G	G. .
E2HR 007	. . . . .					.G	
G2HR 007	. . . . .					.G	
H2HR 007	. . . . .						
B1HR 010	. . . . .					.G	
F1HR 010	. . . . .						
A1HR 010	. . . . .						
1HR 0101	. . . . .						G. .
1HR 0100	. . . . .					.G	G. .
--2HR 0010	. . . . .					.G	
D2HR 010	. . . . .						
B2HR 010	. . . . .					.G	G. .
--2HR 010	. . . . .					.G	
C2HR 010R	. . . . .					.G	G. .
C2HR 010	. . . . .						
G1HR 012	. . . . .					.G	

Sequence	311 315	320	330	340	350	360	370
E1HR 012	AACCAGGTGTTTCGATATGCAAAGAAGGCAGGGCGAGCTACATTAACAAACCC - AAGAT						
B1HR 012	. . . . .						
C1HR 012	. . . . .					G	A. .
D1HR 012	. . . . .						A. .
--2HR 012	. . . . .					G	A. .
C2HR 012	. . . . .						A. .
E2HR 012	. . . . .						A. .
D2HR 012	. . . . .					G	
B2HR 012	. . . . .						A. .
C1Hvirginica	. . . . .		G.				A. .
B1Hvirginica	. . . . .						A. .
A1Hvirginica	. . . . .					G	A. .
D1Hvirginica	. . . . .					G	A. .
E1Hvirginica	. . . . .					G	A. .
AHnaniflora	. . . . .						
DHnaniflora	. . . . .						
CHnaniflora	. . . . .						A. .
GHnaniflora	. . . . .						A. .
EHnaniflora	. . . . .						
C1Hlewisii	. . . . .						A. .
B1Hlewisii	. . . . .					G	
A1Hlewisii	. . . . .						
E1Hlewisii	. . . . .						A. .
D1Hlewisii	. . . . .						
EHshuttlew	. . . . .						A. .
HHshutttlew	. . . . .					G	A. .
GHshutttlew	. . . . .						A. .
DHshutttlew	. . . . .						A. .
CHshutttlew	. . . . .					G	A. .
AHminor	. . . . .						A. .
BHminor	. . . . .						
CHminor	. . . . .						A. .
DHminor	. . . . .						
EHminor	. . . . .						
DHheterophylla	. . . . .						A. .
EHheterophylla	. . . . .						
FHheterophylla	. . . . .					G	A. .
GHheterophylla	. . . . .					C	A. .
HHheterophylla	C.					G	A. .
CHspeciosa	. . . . .						
EHspeciosa	. . . . .						A. .
FHspeciosa	. . . . .						A. .
GHspeciosa	. . . . .						A. .
HHspeciosa	. . . . .						A. .
DHariCallifolia	. . . . .						A. .

Sequence	311 315	320	330	340	350	360	370
EHariCallifolia	AACCAGGTGTTTCGATATGCAAAGAAGGCAGGGGCGAGCTACATTAACAAGCCC - AAGAT						
FHariCallifolia	. . . . .					A.	
GHariCallifolia	. . . . .					A.	.A . .
HHariCallifolia	. . . . .					A.	.A . .
BHariRuthii	. . . . .					A.	.A . .
CHariRuthii	. . . . .					A.	
DHariRuthii	. . . . .					A.	.A . .
EHariRuthii	. . . . .					A.	.A . .
FHariRuthii	. . . . .					A.	
BHariArifolia	. . . . .					A.	.A . .
CHariArifolia	. . . . .					A.	.A . .
DHariArifolia	. . . . .						.A . .
EHariArifolia	. . . . .					A.	
FHariArifolia	. . . . .					A.	
AHeterotropa	. . . . .		G.			A.	.A . .
BHeterotropa	. . . . .		G.			A.	.A . .
CHeterotropa	C.						.A . .
DHeterotropa	. . . . .		G.			A.	.A . .
EHeterotropa	. . . . .		G.			A.	.A . .
FHeterotropa	. . . . .					A.	
A1 A.canadense	. . . . .		G.			A.	
B1A.canadense	. . . . .		G.				.A . .
C1A.canadense	. . . . .		G.			A.	.A . .
E1A.canadense	. . . . .		G.			A.	.A . .
D1A.canadense	. . . . .		G.			A.	.A . .
AIsotrema	. . . . .	CA . G . . C . . G		ATG	T . . C	A.	.A . .
FIsotrema	. . . . .	CA . G . . C . . G		ATG	T . . C	A.	.A . .
CIsotrema	. . . . .	CA . G . . C . . G		ATG	T . . C	A.	.A . .
BIsotrema	. . . . .	CA . G . . C . . G		ATG	T . . C	A.	.A . .
DIsotrema	. . . . .	CA . G . . C . . G		ATG	T . . C	A.	

Sequence	371	380	390
B1HC 001	GCGCCA - TTACGTCGGCAGG		
C1HC 001	. . . . .		
E1HC 001	. . . . . C . . T.		
D1HC 001	.A . . . . . C . . T.		
F1HC 001	. . . . . C		
A2HC 001	.A . . . . . C		
D2HC 001	. . . . . C . . T.		
E2HC 001	. . . . .		
F2HC 001	.A		
G2HC 001	. . . . . C.		
A1HC 003	. . . . .		
B1HC 003	.A . . . . . T.		
C1HC 003	.A . . . . . T.		
F1HC 003	. . . . . T.		
G1HC 003	. . . . . C . . T.		
A2HC 003	.A . . . . . C		
B2HC 003	. . . . . C		
C2HC 003	. . . . .		
D2HC 003	. . . . .		
E2HC 003	.A . . . . . C . . T.		
B2HC 004	. . . . .		
C2HC 004	.A		
A2HC 004	.A . . . . . C.		
D2HC 004	.A . . . . . C. . T.		
--2HC 004	.A . . . . GC.		
E2HC 004	.A		
B3HC 004	.A . . . . . C. . T.		
C3HC 004	. . . . . C.		
D3HC 004	. . . . . T.		
E3HC 004	.A . . . . . C. . T.		
F3HC 004	.A . . . . . C.		
A1HC 006	. . . . . C . . T.		
B1HC 006	.A		
B1HC 006	. . . . . C . . T.		
B1HCR006	.A		
B1HC 006	. . . . .		
C1HC 006	.A . . . . . C.		
D1HC 006	. . . . .		
H1HC 006	. . . . . C.		
F1HC 006	. . . . .		
E1HC 006	. . . . . C.		
--2HC 006	.A		
E2HC 006	.A . . . . . C. . T.		
--2HC 006	.A . . . . . C.		
D2HCr006	. . . . .		

Sequence	371	380	390
C2HC 006	GCGCCA - CTATGTCGGCAGG		
A1HC TN	. . . . . T . . C		
C1HC TN	. A		
D1HC TN	. . . . .		
E1HC TN	. A . . . . . C		
F1HC TN	. A . . . . . C		
C2HC TN	. . . . . C		
D2HC TN	. . . . . T		
F2HC TN	. . . . .		
G2HC TN	. . . . . T		
H2HC TN	. . . . .		
C1HR 004	. . . . . T . . C.		
G1HR 004	. . . . .		
A1HR 004	. A		
E1HR 004	. A		
D1HR 004	. . . . .		
G2HR 004	. . . . . T . . C.		
D2HR 004	. . . . .		
E2HR 004	. . . . . T		
H2HR 004	. . . . .		
C2HR 004	. . . . .		
F2HR 004	.A . . . . T . . C.		
C1HR 007	.A . . . . T . . C.		
A1HR 007	.A . . . . T . . C.		
B1HR 007	. . . . . C.		
D1HR 007	. . . . . C.		
E1HR 007	. . . . . T . . C.		
A2HR 007	.A . . . . . C.		
B2HR 007	. . . . . T . . C.		
C2HR 007	.A . . . . . C.		
E2HR 007	. . . . . C.		
G2HR 007	. . . . . T.		
H2HR 007	. . . . . C.		
B1HR 010	. . . . . C.		
F1HR 010	.A		
A1HR 010	. . . . . T . . C.		
1HR 0101	.A . . . . . C.		
1HR 0100	. . . . . C.		
--2HR 0010	. . . . . C.		
D2HR 010	. . . . . T . . C.		
B2HR 010	. . . . . T . . C.		
--2HR 010	. . . . . C.		
C2HR 010R	. . . . . T . . C.		
C2HR 010	. . . . . C.		
G1HR 012	.A.		

Sequence	371	380	390
E1HR 012	GCGCCA - TTACGTCGGCAGG		
B1HR 012	.....	.....	
C1HR 012	.....	C.....	
D1HR 012	.....	C.....	
--2HR 012	.....	...T.....	
C2HR 012	.....	C.....	
E2HR 012	.....	C.....	
D2HR 012	.....	C.....	
B2HR 012	.....	.....	
C1Hvirginica	.....	C..T.....	
B1Hvirginica	.....	C.....	
A1Hvirginica	.....	...T.....	
D1Hvirginica	.....	C..T.....	
E1Hvirginica	.....	C.....	
AHnaniflora	.....	...T.....	
DHnaniflora	.....	...T.....	
CHnaniflora	.....	C.....	
GHnaniflora	.A.....	C..T.....	
EHnaniflora	.....	C..T.....	
C1Hlewisii	.....	C.....	
B1Hlewisii	.....	C.....	
A1Hlewisii	.....	.....	
E1Hlewisii	.A.....	C.....	
D1Hlewisii	.....	C. -.....	
EHshuttlew	.....	.....	
HHshutttlew	.A.....	.....	
GHshutttlew	.....	.....	
DHshutttlew	.A.....	C..T.....	
CHshutttlew	.A.....	.....	
AHminor	.A.....	C.....	
BHminor	.A.....	...T.....	
CHminor	.A.....	.....	
DHminor	.....	C.....	
EHminor	.A.....	.....	
DHheterophylla	.A.....	C.....	
EHheterophylla	.A.....	...T.....	
FHheterophylla	.....	C.....	
GHheterophylla	.....	C.....	
HHheterophylla	.....	...T.....	
CHspeciosa	.A.....	C.....	
EHspeciosa	.A.....	.....	
FHspeciosa	.....	C.....	
GHspeciosa	.....	...T.....	
HHspeciosa	.....	C.....	
DHariCallifolia	.....	C.....	

BIOGRAPHICAL SKETCH

Joy VanDervort-Sneed was born on January 23, 1981, in Austin, Texas to Linda VanDervort and Reuben Sneed. She graduated from Boulder High School in Colorado in January 2000. The following autumn, she entered Warren Wilson College. During the four years at Warren Wilson College she gained experience in the conservation community by co-coordinating the internship program for the Environmental Leadership in 2003-2004 after having two summer internships with them. The first internship was in Belize for the Programme of Belize, the second was with The Nature Conservancy in Bat Cave, NC. During her last year of college, she was the Resident Director of an all female dorm at Warren Wilson College. She graduated with a Bachelor's of Science degree in Conservation Biology in May 2004. After graduation she worked in the conservation field for one year. At the beginning of 2005 she accepted a teaching assistantship from Appalachian State University. She graduated with her Master's of Science in Biology in May 2008 from Appalachian State University.